THE INFLUENCE OF TOXIC NITROGENOUS COMPOUNDS IN CANINE AND FELINE DIETS ON NITROGEN RETENTION AND CARDIOVASCULAR FUNCTION.

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ABSTRACT

Pet nutrition is a critical part of maintaining a healthy and happy companion animal. In a multibillion dollar industry, there are many types and brands of pet food to choose from. With protein being one of the major macronutrients that make up pet food, it is one of the primary factors conscientious owners consider when feeding their animals. The purpose of this thesis was to analyze protein quality in pet food and consider the utilization of non-protein nitrogen as a therapeutic agent on canine cardiovascular function. Two studies were conducted to research these objectives. The first study analyzed the protein inclusion and screened for toxic nitrogenous compounds in commercial pet foods. Additionally, cardiovascular function in relation to nitrate and nitrite content, was analyzed in dogs fed the same commercial diets. The second study used a cost versus benefit analysis to examine the use of dietary nitrate and nitrite in dog food in order to determine whether it acts a toxic versus therapeutic additive. The results of the first study determined that while protein content in commercial diets increased in proportion to price, protein quality was still similar among commercial diets. Furthermore, diets containing a higher crude protein content also contains higher concentrations of non-protein nitrogen. Lastly, the current nitrate and nitrite content of commercial pet food is not high enough to have any influence of cardiovascular function. The results of the second study reveal that there are cardiovascular differences in dogs fed dietary nitrate versus dietary nitrite. Dietary nitrite showed signs of being potentially therapeutic in lowering blood pressure, with increases in nitrite producing a higher flow mediated dilation and lower heart rate in dogs. In contrast, dietary nitrate showed more cardiotoxic indicators, with increasing nitrate showing no improvements in flow mediated dilation and instead showing increases in heart rate and stroke volume. In conclusion, the results of this thesis show that consumers do not need to spend money on high priced diets, where protein is concerned. In addition, dietary nitrite showed more potential as therapeutic vasodilator in dogs than nitrate.

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LIST OF ABBREVIATIONS

AA- Amino Acids

AAFCO- Association of American Feed Control Officials

ACU- Animal Care Unit

ATSDR- Agency for Toxic Substances and Disease Registry

BUN- Blood Urea Nitrogen

cGMP- cyclic Guanylate Monophosphate

CRP- C-Reactive Protein

ELISA- Enzyme-Linked Immunosorbent Assay

FDA- Food and Drug Administration

FMD- Flow Mediated Dilation

HbO_2- Oxyhemoglobin

MER- Maintenance Energy Requirements

MetHb- Methemoglobin

MLCK- Myosin Light Chain Kinase

NO- Nitric Oxide

ROS- Reactive Oxygen Species

1.0 INTRODUCTION

1.1 Overall Preface

This thesis includes two research chapters written in manuscript style formatting. Chapter 3 will be submitted for publication to the *Journal of Animal Science* and Chapter 4 will be submitted for publication to the *Journal of Animal Physiology and Nutrition (Japan).*

1.2 Rationale

Pet owners exercise a great amount of care when it comes to their animals. Currently there is consumer debate about which pet food brands provide animals with the best nutritive value. With so many different brands available, owners are often overwhelmed with the decision to pick a food that could so greatly influence the overall health of their pet. As a major requirement of companion animals, owners often base their decision on the protein content and apparent 'quality' of ingredients used in different pet food brands. The general trend in thinking is that higher protein, more natural and thus more expensive food is better and healthier. However, with a high animal protein inclusion in pet food, more preservatives must be added to reduce microbial degradation. Inorganic nitrogenous compounds may be used as a preservative in these diets, but this also raises the apparent crude protein content, in a process known as nitrogen doping. Conversely, nitrogenous compounds in the diet, such as nitrite and nitrate have the potential to promote vascular distensibility and vasodilation, through conversion to nitric oxide. While there is a limit for nitrite, no limit is set for nitrate in pet food. Thus, this research attempts to investigate a cost verses benefit analysis of this compound when fed to pets. As a multibillion dollar industry, it is important to ensure the pet food being distributed to consumers will promote and maintain optimal pet health.

1.3 Research design

The purpose of the first research chapter (Chapter 3 in this thesis) was to evaluate the protein inclusion and quality used in commercial pet food. A range of brands and price points were selected to determine differences in the utilization and digestibility of protein after a short-term feeding. In addition, due to previous incidents where pet food was purposefully

contaminated with toxic nitrogenous substances in order to increase the apparent protein content, the same pet foods were analyzed for inorganic nitrogenous compounds and whether or not the inclusion of those compounds reflected the crude protein content. It was also examined if the dietary nitrate and nitrite in the selected commercial diets could be used therapeutically to improve cardiovascular health. A combination of echocardiography and flow mediated dilation was used to analyze the effects of feeding these diets had on cardiac function and vascular distensibility. Lastly, plasma samples collected after feeding were analyzed for biomarkers of toxicity.

The second research chapter (Chapter 4 in this thesis) explores whether the addition of supplementary nitrate and nitrite to canine diets have a therapeutic or toxic effect. Canine diets were first formulated for a crude protein content of 18%, to which plant based or pure nitrate or nitrite was included. To a control diet, supplementary nitrate or nitrite was added via an organic source, as beet pulp or peas, versus inorganic sources, as sodium nitrate and sodium nitrite. Similar to the first research chapter, the cardiovascular health of dogs fed the five diets was analyzed using echocardiography and flow mediated dilation after a short-term feeding. Nitrate and nitrite analysis was also conducted for each of the diets and plasma collected after feeding. Plasma samples were also analyzed for biomarkers of toxicity.

1.4 Objectives

The overall objective of this research was to provide an in-depth analysis of protein used in commercial pet food and determine if nitrate and nitrite added to pet food will have therapeutic or toxic cardiovascular effects in dogs. Specific objectives of each chapter are as follows:

Chapter 3

Objective 3.1 Evaluate if price of commercial pet food influences nitrogen retention and total tract digestibility in dogs and cats.

Objective 3.2 Determine if toxic non-protein nitrogenous compound concentrations differ among commercial diets and how much of it is absorbed and excreted.

Objective 3.3 Investigate if nitrate and nitrite in different commercial diets will produce a change in cardiovascular function.

Chapter 4

Objective 4.1 Investigate if nitrate as a doping agent, has the ability to increase crude protein content.

Objective 4.2 Conduct a cost verses benefit analysis in diets formulated with supplementary nitrate and nitrite.

Objective 4.3 Determine if a limit for nitrate inclusion should be implemented in the pet food industry.

1.5 Hypotheses

The overall hypothesis for this research was that protein quality and utilization would be similar among all commercial diets and that with increasing nitrate and nitrite levels, there would be improved vascular distensibility, without change in biomarkers of subclinical toxicity.

Chapter 3

Hypothesis 3.1 Nitrogen retention and protein quality will be similar among all diets.

Hypothesis 3.2 Diets containing more preservatives and beet pulp will contain a higher concentration of nitrogenous compounds.

Hypothesis 3.3 Commercial diets containing the highest concentrations of nitrate and/or nitrite will have improved vascular distensibility, without changes in cardiac function, nitrotyrosine or methemoglobin.

Chapter 4

Hypothesis 4.1 After a proximate analysis, diets will have a higher crude protein percentage than what was formulated.

Hypothesis 4.2 Dogs will show improved vascular distensibility and lower blood pressure after being fed diets with increasing nitrate and/or nitrite.

Hypothesis 4.3 After being fed diets with supplemental nitrate, dogs will show signs of subclinical toxicity, prompting the need for a maximal nitrate limit in the pet food industry.

2.0 LITERATURE REVIEW

2.1 Companion animal protein requirements

Protein is one of six essential nutrients required for an animal to survive. Proteins are constructed from twenty-two amino acids (AA) which are used for growth and metabolism, with all amino acids containing an amino group, partially comprised of nitrogen. Varying types and configurations of amino acids are included into different types of proteins, giving each protein source a unique nutritional value (Jurgens 2002).

All animals can synthesize twelve of these amino acids, deeming them as “non-essential”. Conversely, certain AAs or “essential” amino acids must be ingested by the animal to perform normal physiological functions. The ten essential amino acids for both cats and dogs include arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Hand et al. 2000). Cats specifically require an eleventh essential amino acid, taurine, at approximately 500 mg/kg of diet dry matter. Insufficient taurine supplementation in cats can lead to retinal degeneration and blindness. For this reason, commercial cat foods are often supplemented with excess taurine (Hayes et al. 1989). Furthermore, insufficient taurine levels in the diet has been linked to dilated cardiomyopathy in both cats and dogs. A study by Fascetti et al. 2003 examined association between a taurine deficient diet and early clinical signs of dilated cardiomyopathy in dogs. Results showed that rice bran and whole rice commercial diets contained the lowest concentrations of taurine. Additionally, it was noted that as a result of being fed the taurine deficient diets, 10 of the 12 dogs used in the study developed echocardiogram abnormalities, with left ventricular enlargement being the most common. These results go against the notion that taurine is not an essential AA in canine diets.

Dogs and cats can utilize protein from multiple sources including mammals, fish, plants and even insects (Bednar et al. 200). There are a variety of protein sources that are added to pet food diets. Of these, beef, chicken, pork and fish are the most common animal protein feedstuffs, while soybean, peas and lentils are the most common types of plant protein feedstuffs (Spitze et al. 2003). All of these proteins have different qualities that can influence the physiological state

of an animal. Each protein sources contains a different configuration of AAs, fat content and palatability (Dust et al. 2007).

Bioavailability and digestibility are two important factors when considering protein sources in pet food. Digestibility is the proportion of ingested food that is broken down in the digestive tract and absorbed into the body (Stein et al. 2007). Meanwhile, bioavailability is the degree to which a specific ingested nutrient is absorbed into the body (Gibson 2007). The term biological value is then used to define the measure of protein that is available to be absorbed (Becker 2007). The biological value of protein is dependent upon the composition and quantity of AAs. Protein from animal sources such as muscle and organ tissue are typically more digestible than those from plant or insect sources, regardless of their overall protein content (Schaafsma et al. 2000). A study by Faber et al. (2010) reviewed the relative digestibility and composition of AAs in beef, chicken, pork, pollock, and salmon. When these proteins were fed to dogs cannulated at the ileum to facilitate sampling of the digesta at the point immediately after absorption, it was determined that pollock contained the highest percentage of total essential amino acids, total nonessential amino acids and the highest total tract digestibility, followed by salmon, beef, pork and chicken respectively.

Different protein sources contain varying percentages of nitrogen, with the crude protein content of feed being determined using industry-standard methods that indirectly measure protein and instead measure the total nitrogen content of the feed (Thiex et al. 2002). This can be a misleading determination of total protein, as some of the nitrogen that is measured would be classified as “non-protein nitrogen” that an animal cannot always use (Li et al. 2015). Some of these non-protein nitrogen sources are derived from organic plant sources that are naturally high in non-protein nitrogen compounds, while other sources might be an intentional or unintentional addition of toxic inorganic additives that contain nitrogen (MacMahon et al. 2012).

Protein requirements for dogs and cats varies between these species. According to the Association of American Feed Control Officials (AAFCO 2013), the average adult dog requires approximately 18% dry matter crude protein in the diet. Cats require a slightly higher protein content at 26% dry matter crude protein. (AAFCO 2013). This is not an exact measurement for cats and dogs as there is variability within each species. There are various confounding factors that influence required protein, such as age, health status, performance requirements, and reproduction status, among others (Dzanis 2014).

2.2 Companion animal cardiovascular pathology

Companion animals and humans share many of the same cardiovascular traits, which means that they are susceptible to many of the same cardiovascular illnesses (Taylor et al. 2004). However, there are certain cardiovascular diseases that can affect certain sizes and breeds more than others. For instance, congestive heart failure is most commonly associated with small dog breeds due to degeneration of the mitral valve (Parker and Kilroy-Glynn 2012). Conversely, large breed dogs are more prone to developing dilated cardiomyopathy, observed as an increase in left end diastolic volume and a decrease in ejection fraction as a result of wall thinning (Stabej et al. 2005). Cats, while still prone to cardiovascular diseases and having a tendency to develop hypertension and hypertrophic cardiomyopathy, are less sensitive than dogs (Saunders 2012). An exception to this in cats was their tendency to develop dilated cardiomyopathy prior to recognition of their dietary requirement for taurine (Pion et al. 1992). Many factors can influence the development of cardiovascular disease in pets including, genetics, exercise, nutrition, breed, parasitic infection and environmental exposure to pathogenic microbes and toxicants (Brown et al. 2007).

Unlike cats and humans, hypertension is less prevalent in dogs. Approximately 10% of dogs develop hypertension, with most of them being senior animals (Remillard et al. 1991). Canine hypertension is diagnosed when systolic pressure exceeds 160 mmHg. Secondary hypertension is much more prevalent in dogs than primary hypertension. Secondary hypertension means that there is usually an underlying disease that causes high blood pressure (Serres et al. 2006). For instance, 93% of dogs that develop renal failure also have high blood pressure (Brown et al. 2007). Obesity is another major contributor to hypertension in dogs, which can be linked to nutrition (Hall et al. 2000). Hypertensive conditions can trigger many pathological conditions including stroke, peripheral vascular disease, kidney disease, pulmonary embolism, and neuropathy, among others (Carmeleit and Jain 2000).

2.3 Nitrogen doping in pet food

Nitrogen doping is the practice whereby non-protein nitrogen is added to animal and human diets to improve the apparent nitrogen content of the food (Li et al. 2015). Non-protein nitrogen is a constituent that may be added to the diet to boost apparent values during analysis

for protein. In reality, the nitrogen from non-protein nitrogen is neither retained nor used by cats and dogs (Ammerman et al. 1995). This could include nitrogen from organic or inorganic sources. In contrast, the incorporation of non-toxic non-protein nitrogen into ruminant diets has been common practice for many years because ruminant microflora utilize non-protein nitrogen to make amino acids (Khan et al. 2015). Unlike ruminants, monogastric animals do not have the proper gut microbial population to convert non-protein nitrogen into usable nitrogen sources (Columbus and de Lange 2012). An example of an organic source of nitrogen would be the naturally occurring non-protein nitrogen amino acids found in plants. These include, but are not limited to L-ornithine, L-homoserine, and L-S-adenosylmethionine, which are intermediates of plant metabolism. While not found in all plants, many of these non-protein nitrogen species are found in seeds and legumes which may be added to the diet (Huang et al. 2011). Conversely, inorganic and possibly toxic nitrogenous compounds in the diet may also boost the apparent protein content. These compounds may include nitrite, nitrate, ammonia and urea (Ahmed et al. 2007). While these compounds have the potential to increase apparent crude protein content, they are not always added for this purpose. It is common practice in pet food manufacturing to incorporate these potentially toxic compounds into the diet as preservatives and to stabilize colour and flavor (Koller 2000).

Previously, other toxic substances have been confirmed as pernicious doping. A widely disregarded example of toxic nitrogenous compound doping is the 2007 melamine crisis. Melamine is a nitrogen-containing compound commonly used in plastic manufacturing (Lund and Peterson 2006). In mid 2007 the United States Food FDA enforced a recall on over one hundred pet food brands as result of positive tests for traces of melamine after an outbreak of toxicity in pets (Skinner et al. 2010). Even with a recall on pet food, many animals had already shown symptoms of toxicity or died. Melamine is a nephrotoxin that causes renal failure by way of urolithiasis. Other noted symptoms of toxicity were lethargy, vomiting, diarrhea, anorexia and in some cases, death (Thompson et al. 2008). It was determined that wheat germ from a Chinese distributer had been tainted with melamine to increase the apparent nitrogen content (Zhou et al. 2012). A large number of different North American pet food manufacturers then used this feed ingredient without knowledge that it had been intentionally tainted, leading to widespread recalls (Cianciolo et al. 2008).

2.4 Nitrate and nitrite toxicity

Nitrate/nitrite toxicity is a common and well-known problem in the livestock industry, with grazing cattle succumbing to disruptions in systemic function and death (Ozmen et al. 2005). Unfortunately, nitrate toxicity is an issue to be wary of for all animals, including humans and pets. When nitrate is ingested, it is converted into the toxic nitrite metabolite by the gut microbiome. Once in the toxic form, it can cause adverse effects on multiple organ systems (Gupta 2012).

The most critical result of excessive nitrate/nitrite exposure is methemoglobinemia, a condition by which oxygen does not properly bind to hemoglobin in erythrocytes (Dorman et al. 2002). Hemoglobin is an iron containing porphyrin ring structure within the red blood cells throughout the cardiovascular system. The function of hemoglobin is to bind oxygen, which can then be transported to different tissues throughout the body (Coates 2014). The oxygen carrying iron in the porphyrin ring is normally in the Fe^{2+} state (Storz 2007). Nitrite can act both directly and indirectly to disrupt hemoglobin formation. Nitrite can act directly on the heme ring by oxidizing the iron, or it can cause the release of free radicals that then in turn oxidize the iron. Once oxidized, the iron in hemoglobin changes from the Fe^{2+} state to the Fe^{3+} state and results in methemoglobin formation (Umbreit 2007, Cavaiuolo et al. 2014). As a result of these physiological changes, metabolic acidosis and gastroenteritis begins to set in. Some of the notable symptoms of methemoglobinemia include diarrhea, dehydration, vomiting, lethargy, coma and death (Okonjo et al. 2014). Figure 2.1 demonstrates how ferrous iron at the hemoglobin core undergoes oxidation through Fenton chemistry, resulting in the formation of methemoglobin with a ferric iron core. The oxidation of iron in red blood cells reduces oxygen carrying capacity and results in the formation of reactive oxygen species. Figure 2.2 illustrates the oxidation of hemoglobin as a result of direct nitrite interference. Nitrite initially diffuses into the blood stream and immediately oxidizes hemoglobin, producing nitric oxide (NO) and methemoglobin (MetHb). Nitrite also reacts with oxyhemoglobin (HbO_2) to produce MetHb. Furthermore, the NO produced can react with HbO_2 to cause additional hemoglobin oxidation into MetHb and nitrate. Nitrate is then further oxidized into nitrite and NO, continuing the cycle.

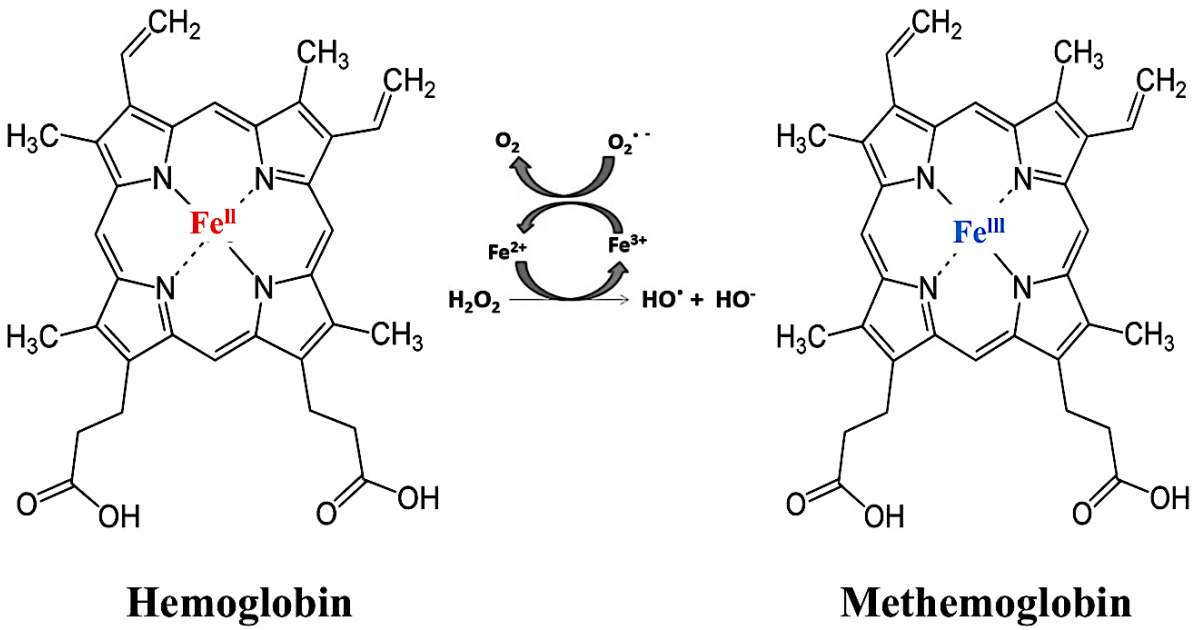


Figure 2.1 Oxidation of hemoglobin to methemoglobin. O_2 = normal state of oxygen; $O_2^{\bullet -}$ = oxygen free radical; H_2O_2 = hydrogen peroxide; Fe^{2+} = reduced state of iron; Fe^{3+} = oxidized state of iron; $HO^{\bullet}$ = hydroxyl radical; HO^- = hydroxide anion.

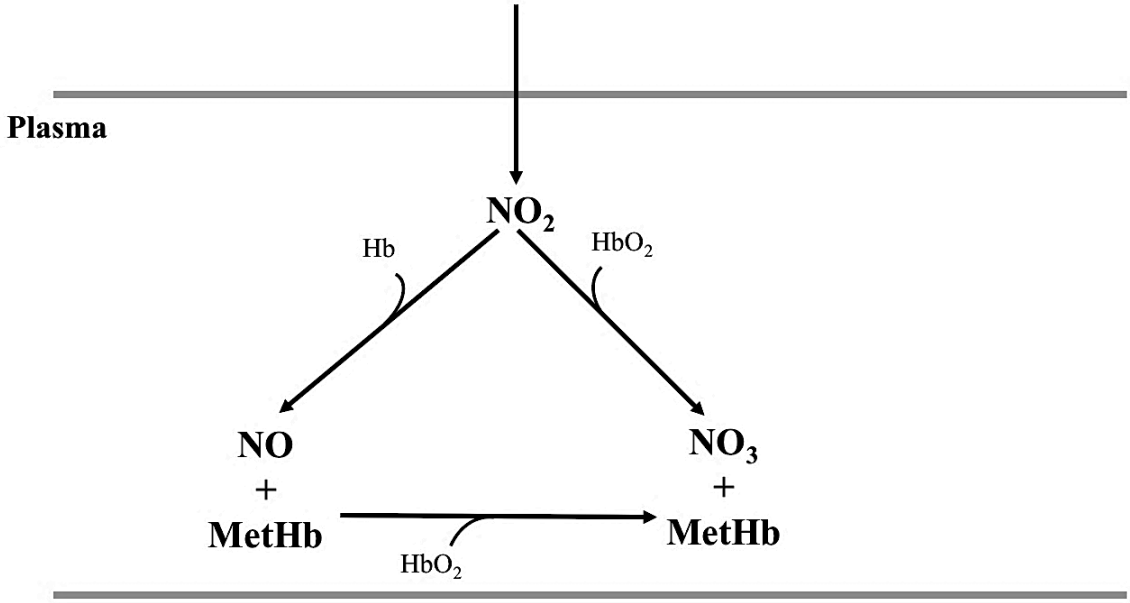


Figure 2.2 Methemoglobin pathway by reaction with nitrite. NO = nitric oxide; NO_2 = nitrite; NO_3 = nitrate; MetHb = methemoglobin; Hb = hemoglobin; HbO_2= oxygenated hemoglobin.

While the hematological effects are one of the most notable conditions involving nitrite toxicity, multiple systems can also be affected. The cardiovascular system is a major target of nitrite effects. Nitrite can alter blood flow by causing hypotension through a conversion to nitric oxide (Lundberg et al. 2008). Nitric oxide stimulates guanylyl cyclase to increase smooth muscle cyclic guanylate monophosphate (cGMP) and decrease intracellular calcium, promoting vasodilation of blood vessels and a decrease in blood pressure (Fleming and Busse 2003). At a moderate dose, this nitrite effect could be beneficial, especially in hypertension, but in higher doses would constitute toxicity. Excess changes in blood flow has been connected to reports of headaches, angina, myocardial infarction and cardiac death as a result of chronic exposure to nitrates and nitrites (Ignarro 2000). However, nitrates can also indirectly be the cause of hypertension. Nitrates have the ability to form reactive oxygen species (ROS) such as superoxide. Oxidative damage can lead to elevated blood pressure due to decreased nitric oxide bioavailability, indicated by increased peroxynitrite and nitrogen dioxide levels. ROS can cause alterations in the fluidity of cell membranes, including those of endothelial cells. This can increase sensitivity to vasoconstricting agents, further increasing blood pressure (Gori and Parker

2008). Peroxynitrite reacts with many macromolecules to cause damage, but has a high affinity for tyrosine residues. Therefore, nitrotyrosine is often used as both a marker of oxidative damage as well as an indicator of oxidative inactivation of nitric oxide (Mohiuddin et al. 2006).

Furthermore, nitrate and nitrite exposure elevate the risk for cancer formation. The Agency for Toxic Substances and Disease Registry (ASTDR) classifies nitrates and nitrites under section Group 1A as "probably carcinogenic to humans" (ASTDR 2016). Nitrogenous compounds have the ability to promote cancer formation in multiple locations in the body. For example, the work by Ward et al. (2010) demonstrates that nitrates have the ability to compete with iodine uptake in the thyroid. The researchers also noted that in human populations where nitrates in the water supply exceeded 5 mg/L, there was an increased incidence of thyroid cancer which in turn resulted in an increase in hyperthyroidism. The ASTDR has also reported multiple cases of non-Hodgkin's lymphoma, esophageal, nasopharynx, bladder, colon, stomach and prostate cancer as a result of chronic exposure to nitrites and nitrates (ASTDR 2016).

2.5 Sources of nitrate and nitrite in pet food

Nitrite is a reactive and easily oxidized compound that is commonly used as a preservative in both human and pet food. This preservative is utilized to reduce microbial growth and protein degradation in meat products (Worth et al. 1997). Nitrite is usually added as the curing agent sodium nitrite and is then oxidized into ROS such as nitrous acid, dinitrogen trioxide, and nitric oxide to reduce the growth and persistence of a variety of microbial strains (Cammack et al. 1999). Mühlig et al. (2014) examined the effects of acidified nitrite salts on the stress responses of *Salmonella enterica* serovar Typhimurium, a notorious raw meat contaminant. It was shown that with the addition of acidified nitrite, the cytoplasmic pH of *S.* Typhimurium was significantly decreased. In addition, there was also an increase in the expression of bacterial stress response genes and a reduction in cell proliferation, thus indicating that acidified nitrite lowers the incidence of *S.* Typhimurium contamination in meat. In contrast, a study conducted by Müller-Herbst et al. (2016) tested the effectiveness of acidified nitrite on the proliferation of *Listeria monocytogenes, in vitro*. Due to *L. monocytogenes'* ability to grow at refrigerator temperatures, it can potentially be a very persistent food contaminant. The research showed that with the addition of acidified nitrite, growth and reproduction of *L. monocytogenes* was inhibited. It was determined that the acidified nitrite was able to reduce the incidence of the

bacteria through a combination of lowering the pH below a survivable level and through a down regulation of the genes in the bacteria. In addition to nitrites being used to prevent spoiling of protein products, it is also commonly used as a colour and flavor enhancer in cured meat. Most notably, nitrite is key in preserving the desirable pink or red colour in cured meats (Bázan-Lugo et al. 2012).

Nitrates are also naturally occurring in plants. Plants take up nitrogen from the soil, often in the form of nitrate and store it in the stems, seeds and foliage (Wang et al. 2012). In companion animal diets, plant sources are commonly incorporated in the form of green leafy vegetables, as well as pulses such as peas (Chan 2011). Another major source of plant nitrate in pet food is beet pulp, an ingredient incorporated into most pet food formulas as a source of slowly digestible fibre (Kröger et al. 2017).

2.6 Therapeutic potential of nitrate and nitrite

The bioactivation of nitrate and nitrite to nitric oxide can have a significant effect on cardiovascular and endothelial function. Despite the earlier section that described adverse effects of nitrate and nitrite on cardiovascular function, there may also be therapeutic and beneficial effects of these compounds.

As nitrate and nitrite make their way through the gastrointestinal system, they can be converted into nitric oxide. Nitric oxide then influences blood pressure by acting as a smooth muscle relaxant, activating guanylyl cyclase to produce cGMP, causing vessels to vasodilate and blood pressure to decrease (Foubert et al. 1994). Studies have shown that the vasodilating properties of nitric oxide derived from nitrate may actually be beneficial for those suffering from high blood pressure. A study by Amin et al. (2012) demonstrated the therapeutic potential of sodium nitrite in an animal model of hypertension. Laboratory mice were injected with angiotensin II in order to increase systolic blood pressure. The mice were then treated with a bolus intravenous injection of sodium nitrite and a significant drop in systolic blood pressure was noted. These results illustrate how nitrite can potentially be used to combat hypertension as well as subsequent coronary artery disease and myocardial infarction.

Nitrite may have other beneficial effects in the cardiovascular system beyond blood pressure lowering. A study by Tang et al. (2011) demonstrated that in humans, nitrite can act as a

selective pulmonary vasodilator, which could potentially be used to combat hypoxia and inflammation during pulmonary hypertension.

2.7 Role of gastrointestinal environment on nitrate and nitrite conversion to nitric oxide

Nitrate is a common non-protein nitrogen source found in food. The compound nitrate is considered not toxic on its own but once it enters the gastrointestinal system and is absorbed into the blood stream, nitrate can be converted into toxic metabolites by enzymes and the microbial population in the gut (Moriya et al. 2002). Once nitrate is converted into nitrite and nitric oxide, these compounds have the potential to alter the homeostatic state of the cardiovascular and pulmonary systems, in addition to being carcinogenic (Grosse et al. 2006). In order for this reaction to occur, nitrate needs to be acted on by a nitrogen reductase enzyme, either occurring in the environment or produced by microflora, in an aerobic environment. The reduction of nitrate causes the extraction of an oxygen molecule from the compound, converting nitrate into nitrite (Wong and Fukuto 1999).

The nitrate reaction begins immediately upon ingestion, where nitrate is converted into nitrogenous metabolites by enzymes in the saliva. A combination of microorganisms and a favourable pH makes the mouth the primary place for the nitrate to nitrite conversion (Tennenbaum et al. 1976). It has previously been shown that the optimal conditions for the salivary conversion of nitrate to nitrite is at a pH of 6-7, as nitrate reductase loses its effectiveness above or below this range (Sanchez et al. 2014). Furthermore, the primary nitrate converting microbial species is *Klebsiella pneumoniae,* a gram-negative bacterium which utilizes the oxygen from environmental nitrogenous sources (Gáspár et al. 2005). Approximately 70% of nitrate is converted in the mouth and is then either directly absorbed into the blood stream as nitrite or passed down into the stomach (Sukuroglu et al 2015). It has also been determined that up to 25% of plasma nitrate can be stored in the salivary glands and continually release. The storage of nitrate promotes continuous entero-salivary recycling (Hughan et al. 2017).

As nitrite moves down the gastrointestinal tract and through the circulatory system, it continues to undergo metabolism and change into other toxic forms. In low pH environments, such as certain areas of the gastrointestinal system and mitochondrial intermembrane, nitrite oxidizes into nitrous acid (Moriya et al. 2002). Nitrous acid proceeds to irritate mucosal membranes and initiate bronchiolar constriction (Beckett et al. 1995). Furthermore, in the

circulatory system nitrite can also be converted into nitrogen dioxide. Nitrogen dioxide can exacerbate respiratory conditions, particularly asthma (Gillespie-Bennett et al. 2011). From nitrogen dioxide, nitrite is then decomposed into nitric oxide, a potent vasodilator. Even though a certain percentage of nitric oxide is produced in the gastrointestinal tract, the half-life of the compound is too short and is often converted back into nitrite before it reaches the vasculature (Tang et al. 2011). Finally, nitrite is also further metabolized into free radical species such as superoxide and hydroxyl radicals, which can go on to accelerate the oxidation of hemoglobin and other oxidative damage (Hughan et al. 2017). As nitrite is reduced into nitric oxide in the gut, nitric oxide may produce localized inflammation in the gastrointestinal tract from the production of free radical species (Yu et al. 2015)

Nitrite that is not chemically oxidized in the stomach moves with the digesta into the small intestine, where it is absorbed through the villi endothelium. From the small intestine, nitrite enters the blood stream where it is converted into nitric oxide (Ignarro 2000); see next section for more detail). Thus, only nitric oxide produced in the blood stream from blood nitrite would have sufficient proximity to vascular smooth muscle to cause vasodilation.

2.8 Nitric oxide production and action in the cardiovascular system

Nitric oxide is a vasodilator that can be both toxic and therapeutic. Nitric oxide is introduced into the vasculature either through oxidation of dietary or endogenous nitrite or more often, through endogenous production by vascular endothelial cells. Once in the blood stream, nitric oxide acts on vascular smooth muscle to promote dilation and a reduction in blood pressure (Ignarro 2000).

The production of endogenous nitric oxide from the endothelium is regulated by a variety of physiological stimuli, including shear stress, acetylcholine, histamine and bradykinin (Fleming and Busse 2003). The vascular endothelial cells sense the change in pressure or endothelial receptors are stimulated, which then initiate the production of nitric oxide from endothelial nitric oxide synthase enzyme. This enzyme converts L-Arginine to nitric oxide (Landmesser et al. 2003). Nitric oxide then diffuses into adjacent smooth muscle, resulting in relaxation of the smooth muscle and a decrease in blood pressure. Due to the short 3-6 second half-life, nitric oxide often has a localized effect (Meller et al. 1992).

Nitrite that is absorbed from the diet can also be converted into nitric oxide in the vascular system. Once absorbed, dietary nitrite diffuses from the intestinal epithelial lining and into the plasma where it reacts with deoxyhemoglobin and a proton to produce nitric oxide and methemoglobin. The conversion of nitrite to nitric oxide is exacerbated under hypoxic and acidic conditions (Lundberg et al. 2008).

Once produced, nitric oxide moves from the endothelium into vascular smooth muscle, where it activates guanylyl cyclase to stimulate an increase in the production of cGMP. The cGMP in turn activates protein kinase G that phosphorylates sarcoplasmic reticular Ca-ATPase and myosin light chain kinase (MLCK). These actions increase reuptake of calcium into the sarcoplasmic reticulum and reduce MLCK activity, respectively. Combined, this reduces actin-myosin crossbridge formation and results in the relaxation of vascular smooth muscle and vasodilation (Klabunde 2011).

2.9 Measurements of nitric oxide and cardiovascular function

Changes in cardiovascular function can be detected and monitored through several methods. Flow-mediated dilation (FMD) is used clinically in human medicine to test endothelial function, serving as an indicator of bioavailability of nitric oxide in response to a shear and hypoxia stressor (Thijssen et al. 2010). Artery diameter is measured using a high-resolution vascular ultrasound. FMD is a measure of the difference in artery diameter at baseline and after occlusion of the artery. Once a baseline measurement is obtained, the cuff is inflated for 5 minutes to occlude the artery. After which, the cuff is deflated, resulting endothelium-dependent hyperemic vasodilation (Harris et al. 2010). This endothelium-dependent response can be compared to the vasodilatory response to a pharmacological substance, such as nitroglycerin (endothelium independent) or nitrite (endothelium independent, but dependent on the presence of heme; Raitakari and Celermaje 2000). Occlusion of the distal limb with the blood pressure cuff reduces artery diameter and increases blood pressure. The increase in blood pressure triggers the release of nitric oxide from the endothelial cells and can be measured as an increase in artery diameter. With an increase in systemic nitrite and nitrate, a subsequent increase in nitric oxide cycling and more pronounced change in artery diameter has been observed in humans (Allen, et al. 2009).

Nitric oxide also has subtle influences on the heart as a modulator of β-adrenergic responses. Nitric oxide has been shown to alter calcium handling in cardiac myocytes and have a positive ionotropic effect on the heart, optimising cardiac pump function (Kanai et al. 1997). Echocardiography can be used to visualize cardiac function using two-dimensional, three-dimensional and Doppler sonography to visualize the heart. Echocardiography can be used to measure end-points such as heart rate, end diastolic volume, end systolic volume, stroke volume and cardiac output. Furthermore, echocardiography can also be used to measure ventricular stiffness by velocity of blood flow through atrioventricular (mitral) valves and ventricular filling (Otto et al. 2015).

2.10 Ammonia and urea toxicity

Like nitrate and nitrite, ammonia and urea are also toxic nitrogenous compounds which may be found in pet food products. Ammonia is a compound that is commonly associated with nitrogenous waste but can also be incorporated into food products as an antimicrobial agent. Anhydrous ammonia specifically is used to reduce the incidence of *Escherichia coli* in beef products after processing (Tajkarimi et al. 2008). Similarly, ammonium hydroxide is used in a variety of non-meat products to reduce incidence of pathogenic microbial species (Naveena et al. 2011). During the urea cycle, trace amounts of ammonia in food are usually converted by mammals into a non-toxic form (Nohara et al. 2015). However, at higher levels or during liver dysfunction, ammonia can result in a condition called hyperammonemia. Hyperammonemia presents as an increase in both intracellular and extracellular pH. At elevated levels, ammonia can cause alterations in cellular signaling and membrane potential due to changes in calcium concentrations (Dasarathy et al. 2017). Ammonia has even been shown to cross the blood brain barrier, initiating neural toxicity associated with seizures, ataxia and coma (Arias et al. 2014). In the liver, chronic hyperammonemia has been shown to induce cirrhosis as a result of toxicity to the stellate cells, causing further impairment of ammonia metabolism (Holek 2015). Ammonia can also have a significant effect on vascular function. Chronic hyperammonemia can generate the production of free radicals, indicated by nitrotyrosine and peroxynitrate. These ROS decrease GMP by un-coupling nitric oxide synthase in the vasculature, preventing production of nitric oxide and leading to reduced vasodilation and increased blood pressure (Xia et al. 1998).

Urea is the product of ammonia metabolism in the mammalian liver and is involved in the excretion of nitrogenous waste. Dogs and cats have higher urinary urea concentrations than humans and thus rely on constant urinary excretion (Liu et al. 2011). This can cause systemic urea toxicity in dogs and cats in certain conditions such as dehydration (Cone et al. 1977). Additionally, with renal dysfunction, elevated blood urea nitrogen (BUN) can develop, leading to a condition termed uremia. Uremia can manifest as a host of systemic symptoms, but is most notably characterized by metabolic acidosis and kidney failure (Lim and Kopple 2000). According to Dalal and Goldfarb 2011, during the 2007 melamine crisis, many canine clinical cases were diagnosed with uremia. Thus, high BUN could be caused by protein doping with nitrogenous toxicants that are incorporated into feed.

3.0 ASSESSING PROTEIN QUALITY OF COMMERCIAL PET FOODS

3.1 Preface

Chapter 3 is the first of two studies included in this thesis on protein quality and nitrogen compounds in pet food. Unlike the following study where only dogs were examined, Chapter 3 includes two species; dogs and cats, for some of the work. This chapter explores the protein quality of commercial pet foods over a range of price points, examining the nitrogen retention, digestibility, nitrogenous compound concentration and cardiovascular function associated with being fed each diet.

This chapter has been submitted for publication in the *Journal of Animal Science*, with the following authors listed: Geiger (responsible for 100% of all animal work, all biochemical tests except those where samples were sent to a contract lab for proximate analyses, all data analyses and all writing), Weber (supervised project, helped with study design and editing). Due to technical difficulties with the methods used in cats, feline data from this chapter was not in the submitted publication, but has been included as in this thesis chapter.

3.2 Abstract

Protein is one of the primary macronutrients required for growth and metabolism. However, a portion of the crude protein listed on pet foods may actually be from non-digestible organic nitrogen or potentially toxic inorganic non-protein nitrogen sources. Neither of which are retained nor used by the animal and may result in alterations in physiological functions. To analyze nitrogen retention and screen for non-protein nitrogen, four commercial pet foods and one lab-made diet were evaluated and fed to beagles and cats. During the first trial, diets were coated with a non-digestible marker, chromium oxide. Seven dogs and eight cats were randomly assigned each diet (n=4 for each diet) and fed the chromium coated diets for 48 hours and urine was collected over this time, followed by total marked fecal collection on the subsequent days and plasma collection at the end of the feeding trial. During a second feeding trial, eight dogs (n=8) were fed the same diets for six days, after which echocardiography was completed. Nitrogen retention was calculated based on nitrogen (%) consumed in feed verses nitrogen lost in

feces and urine. Nitrite, nitrate, ammonia and urea concentrations in all samples were determined using commercial assay kits. The amount of nitrogen retained ranged from 93-96% in dogs and 90-91% in cats but did not statistically differ among commercial diets. Protein digestibility ranged from 69-84% in dogs, with the high protein diets having significantly higher than the lab-made and mid-ranged diets (1-way ANOVA: $P<0.05$). The high protein diet also contained the highest concentration of nitrate with subsequent elevations in plasma nitrotyrosine levels. Methemoglobin levels were significantly lower in the high protein diet ($P>0.05$), possible due to the stimulation of methemoglobin reductase. Furthermore, there was a positive relationship between crude protein, crude fat (simple linear regression: $P=0.02$, $R^2>0.6$), price ($P=0.08$, $R^2>0.6$) and caloric density ($P=0.11$, $R^2>0.6$). There were no significant cardiovascular differences between any of the diets ($P>0.05$). Ultimately, this study shows that in commercial diets, price does reflect protein content but that feeding dogs and cats high protein diets for a long period of time, may result in weight gain and oxidative stress.

3.3 Introduction

Protein, as a nitrogen-containing compound, is essential for growth and metabolism in dogs (Dzanis 1994). However, a portion of the apparent crude protein listed on pet food bags may be from non-protein nitrogen sources. Non-protein nitrogen can be found both as organic non-digestible nitrogen from plant sources and as toxic inorganic sources like nitrate, nitrite, ammonia and urea (Li et al. 2015). Incorporated as a meat preservative or bound within plant products, these compounds can have a toxic effect on the animal, leading to methemoglobin formation and oxidative stress (Carriker et al. 2018). Even at subclinical levels, these compounds have the ability to affect physiological processes such as nitrogen retention and digestibility. The 2007 melamine crisis exemplifies the problems associated with doping pet food with non-protein nitrogen. Nephrotoxic melamine was added to wheat germ used in pet food to boost apparent protein content. As a result, several cases of toxicity and death of companion animals were reported, with many pet food brands getting recalled (Cianciolo et al. 2008). AAFCO and the FDA have set nutritional limits in order to avoid this type of toxicity and ensure proper nutritional maintenance in pet foods (AAFCO 2013, FDA 2018). Macronutrient content, including minimum crude protein, is regulated by AAFCO in order to ensure adequate nutritional quality of commercial pet foods (AAFCO 2013). There are no limits set by the FDA for

ammonia, urea and nitrate in pet food. However, the maximum nitrite concentration in pet food cannot exceed 20 ppm (FDA 2018).

Nitrate and nitrite also have a notable influence on the cardiovascular system through conversion into nitric oxide. Once converted, nitric oxide subsequently acts as a vasodilator to increase blood flow throughout the body and decrease blood pressure (Daiber et al. 2019). Thus, dietary nitrate and nitrite could be indirectly linked to improvements in vascular endothelial function (Carlstrom and Montenegro 2019). Nitrate and nitrite have shown to be therapeutic in terms of lowering systolic blood pressure in humans (Jonvik et al. 2016). Beet root is the most common organic vehicle used to deliver nitrate in human nutrition, with inorganic sources such as sodium nitrate and sodium nitrite also being incorporated as dietary sources (Siervo et al 2013). Ultimately, there may be potential for nitrate and nitrite to also be used to lower blood pressure in companion animals.

The purpose of this study was to assess the protein quality of commercial pet foods and screen for toxic nitrogenous compounds. A secondary objective of this study was to assess the therapeutic potential of dietary nitrate and nitrite in commercial pet food on the cardiovascular system, namely effects on cardiac function, blood pressure and vascular distensibility (flow-mediated dilation). It was hypothesized that due to restrictions in the pet food industry, protein quality and nitrogenous compound concentrations will be similar among all diets. Furthermore, after being fed diets containing ingredients high in nitrate and nitrite, dogs would have improved vascular distensibility without subclinical signs of toxicity. In order to investigate this hypothesis, two different six day feeding studies were conducted using healthy, adult research beagles or mixed breed cats in a randomized cross-over design tested four different commercial diets versus a lab-made diet that all contained chicken as the major protein source. At the end of the first six day feeding study in both cats and dogs, nitrogen retention and protein digestibility were related to dietary, plasma, urine and fecal nitrate, nitrite, ammonia and urea levels. At the end of the second six day feeding study in just dogs, cardiovascular responses were determined using echocardiography, flow-mediated dilation (tests endothelium-dependent relaxation) and high-definition oscillometry (indirect blood pressure). These measurements were then related to dietary, plasma, urine and fecal nitrate or nitrite levels plus blood methemoglobin and plasma nitrotyrosine (indicator of oxidative stress) levels.

3.4 Materials and methods

Chapter 3 was split into two different feeding trials, with different sample sizes but still using the same animals. For the first feeding trial, diets were randomized between animals and the diets were repeated four times (n=4/species) due to availability of metabolic cages. End points of the first feeding trial with a sample size of four include; nitrogen retention, nitrate and nitrite concentrations in all biological samples, and protein digestibility. The second feeding trial utilized all of the dogs, allowing for a sample size of eight (n=8 animals/diet). End points of the second feeding trial with a sample size of eight include; cardiovascular endpoints, ammonia and urea in all biological samples, methemoglobin and nitrotyrosine.

3.4.1 Animals

Companion animal health is the focus of this study, therefore both cats and dogs were used to carry out the research. Eight adult cats (five spayed females and three neutered males) obtained from a certified scientific breeder (Liberty Research, NY) were used for this study. Cats were mixed breed and were 5±1years of age. The cats were housed in the animal care unit (ACU) of the Western College of Veterinary Medicine (Saskatoon, SK). Cats were allowed to roam the facility free during the day, but kept in individual kennels during feeding and overnight. Eight adult beagle dogs (four spayed females and four neutered males) of 5±0.5years of age at the time of this study were originally obtained from a certified scientific breeder (Marshall Farms, NY). Dogs had their own individual kennels for feeding and overnight, but were kept together in open kennels during the day to socialize with each other, with access to outdoor runs and taken on daily walks. When not on trial, dogs were fed a standard commercial adult maintenance pet food diet (Hills Pet Nutrition Inc, Topeka, Kansas, USA). The weight of food fed per animal per day varied for each individual, but portions were adjusted for each animal as needed to maintain ideal body condition score (4-6 on 9-point Purina body condition scale). Dogs and cats were clinically healthy prior to and throughout the study. All procedures and handling involving dogs and cats were completed according to protocol approved by the University of Saskatchewan's Animal Research Ethics Board according to guidelines established by the Canadian Council on Animal Care.

3.4.2 Diet selection

Four commercial pet food brands, Orijen (Champion Pet Foods LP, Edmonton, AB, Canada), Blue Buffalo (Blue Buffalo Co, Wilton, Ct, USA), Purina (Société des Produits Nestlé S.A., Vevey, Switzerland), and Iams (Mars Petcare, Inc., Mclean, VA, USA), were selected based on a spectrum of price points and crude protein content. The same brands were selected for both dogs and cats. All diets were selected for adult animal health maintenance and included similar macronutrients, with chicken as the major protein source. These commercial diets were compared to an experimental diet, formulated in lab for both dogs and cats during previous experiments (Briens et al. unpublished). Feed weights were calculated based on body condition score and body weight, with reference to labelled digestible energy per weight to produce isocaloric portions during testing.

3.4.3 Nitrogen retention and protein utilization

Prior to feeding trials, diets were coated in a non-digestible marker, chromium oxide (VWR, Mississauga, Canada) at 0.01% (w/w) Cr_2O_3 to feed, to aid determination of transit time of the diet and to aid in the determination of protein digestibility (Peachey et al. 2000). At the start of the experiment, meal portions were allocated to each animal based on each individual's history of energy needs to maintain optimal condition and caloric density of the diet, in order to maintain optimal body condition score throughout the trial. During the first feeding trial, seven dogs and eight cats were fed five different diets in a randomized fashion such that each diet was tested in four different animals (n=4/diet/species). Animals were acclimated to the uncoated diets for two days prior to sample collection. After acclimation period, all dogs were housed in individual metabolic cages to allow for total urine collection and fed chromium coated diet for 48 hours (Bingham et al. 2004). Total fecal output resulting from the diet during this 48 hr period was collected based on presence of the non-digestible marker in the feces, with fecal collection extending beyond the 48 hr period as needed until all marked feces had passed. After the 48 hrs, animals were maintained on uncoated test diet and kept alone in their home kennel until all marked feces passed (an additional two days was sufficient; Carciofi et al. 2007). All cats underwent the same feeding regimen but were housed in their own kennels with individual litter boxes throughout the whole trial. Non-absorbable litter was used in their litter boxes to facilitate urine collection. At 96 hr after starting this trial, blood (1.0ml) was collected into EDTA tubes

from a sub-sample of animals (n=4/species), spun at 5000 xg for 10 minutes and plasma aliquoted, then stored at -80°C until use in nitrite/nitrate determination assays. Animals were then returned to regular husbandry. Feed and fecal samples were dried in an oven at 65°C for seven days or until dry, ground and stored at room temperature until analyzed for macronutrients and total nitrogen levels by a commercial laboratory (Central Testing Laboratories, Winnipeg, MB, Canada). Urine was stored at -20°C until total nitrogen analysis (Central Testing Laboratories, Winnipeg, MB, Canada). Nitrogen retention was calculated according to the equation used by Tome et al. 2000, based on feed intake verses nitrogen loss in urine and feces.

Nitrogen retention (NR) was calculated as:

Equation 3.1

$$NR = \frac{\text{total feed intake} - (\text{fecal nitrogen} + \text{urine nitrogen})}{\text{total feed intake}}.$$

Presence of the chromium oxide marker in the feed and feces was determined using atomic absorption spectroscopy (Central Testing Laboratories, Winnipeg, MB, Canada). Protein digestibility was calculated in all of the diets based on presence of the chromium oxide marker (Hernot et al. 2006).

Protein digestibility was calculated as:

Equation 3.2

$$\text{Digestibility (\%)} = 1 - \left[\frac{\%\text{crude protein in feces} \times \%\text{marker in feed}}{\%\text{crude protein in feed} \times \%\text{marker in feces}}\right]$$

3.4.4 Cardiovascular ultrasound

In a second round of feeding trials, eight dogs (but not cats) were fed for six days on each diet in their home kennels, followed by ultrasound testing on dogs fasted overnight, in the morning of day 7. During this second feeding trial, each dog was fed 5 different diets (n=8) in a randomized crossover design. All dogs were previously acclimated to all blood collection and ultrasound procedures by providing positive attention during testing and treats after all procedures were done. Thus, the dogs were highly cooperative and we were able to examine the dogs without stress or any sedation for these procedures. Prior to ultrasound, dogs were weighed and blood pressure was taken using a high definition canine/feline oscillometer (S + B medVET

GmbH, Babenhausen, Germany). Endpoints of flow mediated dilation included brachial artery diameter during baseline, during inflation of a blood pressure cuff placed distal to the brachial artery and at the time of peak dilation (30 seconds) after cuff release previously determined in our group in dogs (Raitkatari et al. 2000; Adolphe et al., 2012). Echocardiography endpoints to asses cardiac function included, heart rate, stroke volume and cardiac output (Otto et al. 2019; Adolphe et al., 2012). Flow mediated dilation and echocardiography were measured using a SonoSite Edge II ultrasound (Fujifilm SonoSite inc., Bothell, USA), with detection using the P10x transducer (8-4 Hz) to detect cardiac endpoints and the L38xi (10-5 Hz) transducer to measure flow-mediated dilation. After ultrasound was conducted, a 3.0 ml aliquot of plasma was obtained for use in ammonia, urea, and nitrotyrosine assays. 1.0 ml aliquot for blood gases was also collected for methemoglobin analysis using a blood gas electrolyte analyzer (Shinova Medical Co., Shanghai, China). Cats were not used for the second feeding trial due to poor resolution of the brachial artery during ultrasound and excessive stress to the cats during the handling needed to make these ultrasound measurements.

3.4.5 Nitrogenous compound and biomarker assays

Plasma, urine, feed and fecal samples were analyzed for nitrite and nitrate in a sub-sample of cats and dogs (n=4/diet/species). Plasma and urine were analyzed directly in the assay, while feed and fecal nitrate and nitrite was extracted into solution. Solid feed and fecal samples were ground and diluted using a 1:10 dilution with reagent-grade water. Diluted samples were heated at 60°C for three hours to extract nitrogenous compounds. All samples were filtered using a 10 kDa cut-off filter to reduce protein interference in the colorimetric assays. Nitrite and nitrate were analyzed using a commercially available nitrite/nitrate assay kit based on the Greiss colour reaction. Nitrite was measured directly and nitrate was calculated based on subtracting nitrite from the total nitrate/nitrite detected. (R&D Systems, Bio-Techne Corporation, Minneapolis, MN, USA). Where a sample nitrate or nitrite levels were below detection, a zero value was used in statistical analyses for that sample. Plasma, urine, feed and fecal samples were analyzed for ammonia and urea in dogs only. Ammonia and urea were determined in the same sub-sample (n=8 animals) using a commercially available urea/ammonia (rapid) test kit (Megazyme, Genzyme, Englewood Cliffs, NJ, USA). Plasma samples from the second dog feeding trial were analyzed for nitrotyrosine. Nitrotyrosine was analyzed using a commercially available

nitrotyrosine enzyme-linked immunosorbent assay (ELISA) (Hycult Biotech, Uden, The Netherlands). C-reactive protein was quantified in plasma samples using a commercially available C-reactive protein bioassay canine enzyme-linked immunosorbent assay (United States Biological, Salem, MA, USA).

3.4.6 Statistical analysis

All data was initially tested for parametric assumptions: a Levene's test was used to test for homogeneity of variance and a KS-test was used to test if data was normally distributed. All data met parametric assumptions. For each feeding trial, endpoints were analyzed independently using 1-way ANOVA followed by Fisher's LSD post hoc tests for pairwise comparisons, with α set at 0.05. Furthermore, linear regressions were used to examine the relationship between the endpoints and crude protein content, with a relationship deemed significant when $R^2>0.6$. A descriptive analysis was used to relate results to price. Data are represented as mean ± SEM. All data analysis was performed using SPSS statistics version 25 (SPSS Chicago, Il, USA, IBM), using linear mixed models.

3.5 Results

All diets were enthusiastically consumed by all of the dogs and cats, with no signs of food refusal or alterations in overall health (based on general appearance and behaviour). Diets are shown arranged in tables and figures from highest to lowest price per kilogram and crude protein percentage, with Orijen being the highest and Purina being the lowest.

3.5.1 Guaranteed and proximate analysis

After a descriptive analysis, this study determined that all commercial diets met and/or exceeded the minimum AAFCO nutritional protein and metabolizable energy requirements for dogs and cats (AAFCO 2013), with crude protein guaranteed analyses ranging from 18-38% in dog foods and 30-40% in cat foods. As shown in Table 3.1 (dog) and Table 3.2 (cat), pet food with a higher price per kilogram also contained higher crude protein, crude fat and caloric density. On average, price per kilogram of cat food was 46% higher than dog food. The commercial pet foods were compared to a lab-made test diet formulated similar to the

commercial brands with chicken as the major protein source and 30% inclusion of pea meal, designated the ‘test pea’ diet (Briens et al., unpublished; Tables 3.1 and 3.2).

Proximate analysis determined that feed crude protein percentage was the same or higher than what the guaranteed analysis stated on the pet food bags for all diets tested except for the Orijen cat food where measured crude protein was lower than the guaranteed analysis (Table 3.3 for dog and Table 3.4 for cat). Despite the lower than guaranteed analysis levels for measured crude protein in the Orijen cat food, the actual level more than exceeded dietary requirements for cats. A descriptive analysis of the diet ingredients (Table 3.3 for dog and Table 3.4 for cat) revealed that diets with a higher price per kilogram used more expensive ingredient sources and a greater number of ingredients. The more expensive manufacturers also used grain-free fibre sources as opposed to corn meal as the primary fibre source used by the less expensive diets.

Table 3.1 Diet description of commercial dog foods.

Diet	Price ($/kg)	Guaranteed analysis				Proximate analysis
		Calories (kcal/kg)	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Crude protein (%)
Orijen	8.50	3900	38	18	4	39
Test pea	N/A	3509	34	15	3.5	34
Blue Buffalo	6.61	3627	24	14	5	30
Iams	3.23	3397	20	9	5	23
Purina	2.08	3407	18	8.5	6	23

Table 3.2 Diet description of commercial cat foods.

Diet	Price ($/kg)	Guaranteed analysis				Proximate analysis
		Calories (kcal/kg)	Crude protein (%)	Crude fat (%)	Crude fibre (%)	Crude protein (%)
Orijen	11.85	4160	40	20	8	39
Test pea	N/A	3509	34	15	3.5	37
Blue Buffalo	10.39	3827	32	15	6	33
Iams	6.28	3710	32	15	3	33
Purina	3.99	3568	30	12	4.5	37

Table 3.3 Ingredient list of five commercial dog foods plus one lab-made test diet (test pea).

Diets				
Orijen	Test pea	Blue Buffalo	Iams	Purina
Chicken meat	Chicken meal	Deboned chicken	Corn meal	Ground yellow corn
Turkey meat	Soy protein concentrate	Chicken meal	Chicken by-product meal	Corn germ meal
Eggs	Chicken fat	Brown rice	Ground whole grain sorghum	Pork and bone meal
Chicken liver	Pea fibre	Barely	Chicken	Tallow preserved with mixed tocopherols
Whole herring	Fishmeal	Oatmeal	Ground whole grain barley	Poultry by-product meal
Whole flounder	Fish oil	Pea starch	Dried beet pulp	Corn gluten meal
Chicken necks	Celite	Flaxseed	Chicken flavor	Animal digest
Chicken heart	Potassium chloride	Chicken fat	Chicken fat	Salt
Turkey heart	DL-methionine	Dried tomato pomace	Dried egg product	Calcium carbonate
Dehydrated chicken	Mineral premix	Natural flavor	Brewers dried yeast	Peas
Dehydrated turkey	Vitamin premix	Peas	Potassium chloride	Potassium chloride
Whole makerel	Taurine	Pea protein	Salt	Natural grill flavour
Whole sardine	Dicalcium phosphate	Salt	Choline chloride	Choline chloride
Whole herring		Potassium chloride	Flax meal	Zinc sulfate
Red lentils		Dehydrated alfalfa meal	Calcium carbonate	Red 40
Green lentils		Potatoes	DL-methionine	Ferrous sulfate
Whole green peas		Dried chicory root	Fructooligosaccharides	DL-methionine
Lentil fibre		Pea fibre	Minerals	Vitamin E supplement
Whole chickpeas		Alfalfa nutrient concentrate	L-lysine monohydrochloride	Manganese sulfate
Whole yellow peas		Calcium carbonate	Vitamins	Yellow 5
Whole pinto beans		Choline chloride	Vitamin B12 supplement	Blue 2
Whole navy beans		DL-methionine	Niacin	Niacin

Table 3.3 Continued.

Diets				
Orijen	Test pea	Blue Buffalo	Iams	Purina
Herring oil		Preserved mixed with tocopherols	Riboflavin supplement	Vitamin A supplement
Chicken fat		Dicalcium phosphate	Inositol	Copper sulfate
Chicken cartilage		Sweet potatoes	Pyridoxine hydrochloride	Calcium pantothenate
Freeze dried chicken liver		Carrots	Vitamin D3 supplement	Garlic oil
Freeze dried turkey liver		Garlic	Folic acid	Pyridoxine Hydrochloride
Whole pumpkin		Zinc amino acid chelate	Dicalcium	Vitamin B-12 supplement
Whole butternut squash		Zinc sulfate	Phosphate	Thiamine mononitrate
Whole zucchini		Vegetable juice	L-tryptophan	Vitamin D-3 supplement
Whole parsnips		Ferrous sulfate	L-carnitine	Riboflavin supplement
Carrots		Vitamin E supplement	Citric acid	Calcium iodate
Whole red delicious apples		Iron amino acid chelate	Rosemary extract	Menadione sodium bisulfite complex
Whole barlett pears		Blueberries		Folic acid
Kale		Cranberries		Biotin
Spinach		Barley grass		Sodium selenite
Beet greens		Parsley		F-5023-C
Turnip greens		Tumeric		
Kelp		Dried kelp		
Whole cranberries		Yucca extract		
Whole blueberries		Niacin		
Whole saskatoon berries		Glucosamine hydrochloride		
Chicory root		Calcium pantothenate		
Turmeric root		Copper sulfate		
Milk thistle		Biotin		
Burdock root		L-ascorbyl-2-polyphosphate		
Lavender		L-lysine		

Table 3.3 Continued.

Diets				
Orijen	Test pea	Blue Buffalo	Iams	Purina
Marshmallow root		L-carnitine		
Rosehips		Vitamin A supplement		
Enterococcus faecium		Copper amino acid		
Zinc chelate		Manganese sulfate		
		Taurine		
		Manganese amino acid chelate		
		Thiamine mononitrate		
		Riboflavin		
		Vitamin D3 supplement		
		Vitamin B12 supplement		
		Pyridoxine hydrochloride		
		Calcium iodate		
		Dried yeast		
		Dried enterococcus faecium fermentation product		
		Dried lactobacillus acidophilus fermentation product		
		Dried aspergillus niger fermentation extract		
		Dried trichoderma longibrachiatum fermentation extract		
		Dried bacillus subtillis fermentation extract		
		Folic acid		

Table 3.4 Ingredient list of five commercial cat foods.

Diets				
Orijen	Test pea	Blue Buffalo	Iams	Purina
Chicken meat	Chicken meal	Deboned chicken	Chicken	Ground yellow corn
Turkey meat	Soy protein concentrate	Chicken meal	Chicken by-product meal	Corn gluten meal
Eggs	Chicken fat	Brown rice	Corn meal	Chicken by-product meal
Chicken liver	Pea fibre	Barely	Brewers rice	Meat and bone meal
Whole flounder	Fishmeal	Oatmeal	Dried beet pulp	Beef tallow preserved with mixed tocopherols
Whole herring	Fish oil	Pea protein	Natural flavour	Soybean meal
Turkey liver	Celite	Peas	Poultry by-product meal	Animal liver flavour
Chicken heart	Potassium chloride	Chicken fat	Dried egg product	Phosphoric acid
Turkey heart	DL-methionine	Dried egg product	Brewers dried yeast	Salt
Chicken necks	Mineral premix	Menhaden fish meal	Sodium bisulfate	Tuna meal
Dehydrated chicken	Vitamin premix	Pea fibre	Fructooligosaccharides	Turkey by-product meal
Dehydrated turkey	Taurine	Natural flavour	Potassium chloride	Salmon meal
Whole mackerel	Dicalcium phosphate	Flaxseed	Choline chloride	Calcium carbonate
Whole sardine		Calcium chloride	Fish oil	Potassium chloride
Whole herring		Fish oil	DL-methionine	Choline chloride
Chicken fat		Choline chloride	Calcium carbonate	Dried cheese powder
Whole red lentils		Potatoes	Vitamins	Dried egg product
Whole green peas		Potassium chloride	Minerals	Taurine
Whole green lentils		Alfalfa nutrient concentrate	Pyridoxine hydrochloride	Minerals
Whole chickpeas		Dehydrated alfalfa meal	Vitamin B12 supplement	Vitamins
Whole yellow peas		Dried chicory root	Riboflavin supplement	Vitamin A supplement
Lentil fibre		Alfalfa nutrient concentrate	Inositol	Calcium pantothenate

Table 3.4 Continued.

Diets				
Orijen	Test pea	Blue Buffalo	Iams	Purina
Whole pinto beans		Taurine	Vitamin D3 supplement	Thiamine mononitrate
Whole navy beans		Calcium carbonate	Taurine	Riboflavin supplement
Chicken cartilage		Salt	Minerals	Vitamin B-12 supplement
Herring oil		Cranberries	Chicken fat	Pyridoxine Hydrochloride
Freeze dried chicken liver		Sweet potatoes	L-carnitine	Folic acid
Freeze dried turkey liver		Carrots	Rosemary extract	Vitamin D-3 supplement
Whole pumpkin		Vegetable juice		Biotin
Whole butternut squash		Ferrous sulfate		Menadione sodium bisulfite complex
Whole zucchini		Niacin		Red 40
Whole parsnips		Iron amino acid chelate		Yellow 5
Carrots		Zinc sulfate		Blue 2
Whole red delicious apples		Vitamin E supplement		Yellow 6
Whole barlett pears		Blueberries		Manganese sulphate
Kale		Barley grass		Calcium phosphate
Spinach		Parsley		Niacin
Beet greens		Tumeric		Blue #2
Turnip greens		Dried kelp		Vit A
Brown kelp		Yucca extract		Peridoxine hydrochloride
Whole cranberries		Copper sulfate		Folic acid
Whole blueberries		Thiamin mononitrate		Vit D3
Whole saskatoon berries		Calcium pantothenate		Calcium iodate
Chicory root		Copper amino acid chelate		Biotin
Turmeric root		L-lysine		Menadione sodium bisulfate complex
Milk thistle		Biotin		Sodium selenite

Table 3.4 Continued.

Diets				
Orijen	Test pea	Blue Buffalo	Iams	Purina
Burdock root		L-carnitine		L-6000-C
Lavender		Vitamin A supplement		
Marshmallow root		Manganese sulfate		
Rosehips		Manganese amino acid chelate		
Enterococcus faecium		Pyridoxine hydrochloride		
		Calcium pantothenate		
		Manganese amino acid chelate		
		Riboflavin		
		Vitamin D3 supplement		
		Vitamin B12 supplement		
		Folic acid		
		Dried yeast		
		Calcium iodate		
		Dried enterococcus faecium fermentation product		
		Dried lactobacillus acidophilus fermentation product		
		Dried aspergillus niger fermentation extract		
		Dried trichoderma longibrachiatum fermentation extract		
		Dried bacillus subtillis fermentation extract		
		Calcium iodate		
		Sodium selenite		

3.5.2 Protein digestibility and nitrogen retention

Protein digestibility differed significantly among diets in dogs (1-way ANOVA; $P<0.05$) and ranged from 68.6-84.2% (Table 3.5). There was no association between protein digestibility and price, as the highest and lowest priced diets showed the highest digestibility. Nitrogen retention of the diets was high in dogs for all diets, with greater than 90% retention for all of the diets. Only the lab-made diet differed significantly from the commercial diets ($P<0.05$) with lower digestibility (Table 3.5).

In contrast, apparent protein digestibility among cat diets did not differ significantly ($P>0.05$) despite the fact that digestibility ranged from 0-80.7% (Table 3.6). Due to the higher fat content of cat diets compared to dogs, we experienced difficulty in coating the diets evenly with the non-digestible chromium marker. Negative digestibility values were removed from analyses, but were frequent in all but the test pea diet. Other values were unreasonably low, but were left in (Table 3.6). Thus, this method used for analyzing digestibility in cats resulted in values that were most likely inaccurate, and the same method is not recommended for future studies in cats. In contrast to digestibility the methods used for nitrogen retention were not affected by the uneven chromium coating. Therefore, similar to dogs, nitrogen retention of the commercial diets was also high in the cats and ranged from 90.5-94.9%, giving confidence to our conclusion that the protein digestibility was not producing real numbers. Despite this, nitrogen retention was not statistically different among the cat diets ($P>0.05$; Table 3.6).

Table 3.5 Protein digestibility and nitrogen retention in dogs fed commercial diets. Diets are listed in decreasing level of crude protein inclusion.

Diet	Protein digestibility (%)	Nitrogen retention (%)
Orijen	84.2 ± 1.0^{a}	93.9 ± 1.0^{ab}
Test pea	75.3 ± 2.8^{b}	92.7 ± 1.4^{b}
Blue Buffalo	82.0 ± 1.7^{a}	96.1 ± 0.3^{a}
Iams	68.6 ± 3.4^{b}	96.3 ± 0.5^{a}
Purina	83.7 ± 1.7^{a}	94.9 ± 0.6^{ab}

Values shown as mean ± SEM, n=4. Values in a column with superscripts without a common letter differ, $P < 0.05$; 1-way ANOVA with LSD post-hoc test.

Table 3.6 Protein digestibility and nitrogen retention in cats fed commercial diets. Diets are listed in decreasing level of crude protein inclusion.

Diet	Protein digestibility (%)[1]	Nitrogen retention (%)
Orijen	48.1 ± 3.2	90.5 ± 1.8
Test pea	80.7 ± 4.2	93.4 ± 0.7
Blue Buffalo	64.0	91.6 ± 1.6
Iams	38.9 ± 3.7	94.5 ± 0.6
Purina	0 ± 0	94.9 ± 0.8

Values shown as mean ± SEM, n=4 for nitrogen retention. No significant differences, P> 0.05; 1-way ANOVA with LSD post-hoc test.
[1]Sample size differs for each group due to the removal of clearly inaccurate values (negative digestibility values). Orijen n=2, Test pea n=4, Blue Buffalo n=1, Iams n=3, Purina n=4

3.5.3 Toxic nitrogenous compounds

Table 3.7 portrays the levels of nitrate and nitrite in dog feed plus plasma, urine and fecal samples. Nitrate in the feed ranged from 2.2-22.8 mg/kg, with diets highest in price and protein having the greatest feed nitrate content. Feed nitrite was similar among all of the diets, ranging from 2.0-3.2 mg/kg. There were no manufacturers tested that exceeded the FDA limit for nitrite of 20 mg/kg (FDA 2018). Canine plasma levels of nitrate were higher than plasma levels of nitrite, following the trends for nitrate and nitrite in the feed (Table 3.7). Only Orijen differed significantly from the other diets and produced the highest plasma nitrate levels in dogs ($P<0.05$; Table 3.7). Dietary nitrate was primarily excreted in the urine in dogs, ranging from 3.4-5.5 μM in urine versus 0.2-1.5 mg/kg in dog feces. Excreted nitrate and nitrite only differed significantly in the feces ($P<0.05$; Table 3.7), with the lab-made diet having significantly greater fecal nitrate and nitrite excretion in dogs compared to all the commercial diets. Moreover, dogs fed Purina had an intermediate level of fecal nitrite, significantly different from the higher test pea diet and all other commercial diets (Table 3.7).

Table 3.8 portrays the levels of nitrate and nitrite in cat feed plus plasma, urine and fecal samples. Feed nitrate ranged from 2.2-87.7 mg/kg, with Orijen containing the highest concentration of nitrate and the test pea diet containing the lowest. Meanwhile, feed nitrite was similar among all diets, ranging from 1.4-3.0 mg/kg. Neither nitrate nor nitrite in the cat feeds followed crude protein levels. There were no manufacturers tested that exceeded the FDA 20 mg/kg limit for nitrite (FDA 2018). Cat plasma levels of nitrate and nitrite were also low after six days of feeding all of the cat diets and did not differ significantly among diets ($P>0.05$; Table 3.8). All plasma nitrate levels were non-detectable and maximum plasma nitrite reached 1.4±0.4 μM in cats. Excretion of nitrate was similarly low in both urine and feces in cats. Fecal and urinary nitrate did not differ significantly among cats after feeding the different diets ($P>0.05$, Table 3.8). In contrast, urine nitrite exceeded the corresponding fecal nitrite for a given diet in cats, ranging from 1.9-4.4 μM nitrite in the urine. Urinary excretion of nitrite was significantly lower in cats fed commercial diets from Orijen and Blue Buffalo compared to all other diets ($P<0.05$; Table 3.8).

Table 3.7 Nitrate and nitrite concentrations in dog feed as well as plasma, urine, and feces after being fed commercial diets or a lab diet (test pea) for six days (plasma and feces) or two days (urine). Diets are listed in decreasing level of crude protein inclusion.

	Feed (mg/kg)		Plasma (μM)		Urine (μM)		Feces (mg/kg)	
Diet	Nitrate	Nitrite	Nitrate	Nitrite	Nitrate	Nitrite	Nitrate	Nitrite
Orijen	22.8	2.5	11.1 ± 3.9^{b}	ND[1]	7.9 ± 5.1	4.2 ± 0.7	0.4 ± 0.1^{a}	$1.7 \pm 0.3^{a,b}$
Test pea	2.2	2.0	3.1 ± 1.6^{a}	ND	1.3 ± 0.6	3.4 ± 1.4	1.5 ± 0.2^{b}	3.3 ± 0.1^{c}
Blue Buffalo	12.8	3.2	0.7 ± 0.5^{a}	ND	7.7 ± 3.5	3.6 ± 0.7	0.2 ± 0.1^{a}	1.3 ± 0.1^{a}
Iams	5.8	2.7	1.9 ± 1.0^{a}	ND	15.7 ± 5.9	3.4 ± 0.5	0.6 ± 0.2^{a}	1.6 ± 0.2^{a}
Purina	7.9	2.5	3.8 ± 1.3^{a}	ND	6.6 ± 2.2	5.5 ± 1.2	0.6 ± 0.2^{a}	2.1 ± 0.1^{b}

Values shown as mean ± SEM, n=4 for plasma, urine and fecal samples. Feed samples are shown as averaged values from duplicate determinations of the same sample. Values in a column with superscripts without a common letter differ, $P < 0.05$; 1-way ANOVA with LSD post-hoc test. Columns without any superscripts showed no significant differences among diets in 1-way ANOVA.
[1]Not detectable (ND)

Table 3.8 Nitrate and nitrite concentrations in cat feed as well as plasma, urine, and feces after being fed commercial diets or a lab diet (test pea) for six days (plasma and feces) or two days (urine). Diets are listed in decreasing level of crude protein inclusion.

	Feed (mg/kg)		Plasma (μM)		Urine (μM)		Feces (mg/kg)	
Diet	Nitrate	Nitrite	Nitrate	Nitrite	Nitrate	Nitrite	Nitrate	Nitrite
Orijen	87.7	1.4	ND[1]	1.1 ± 0.4	5.5 ± 5.5	2.3 ± 0.2[a,b]	0.6 ± 0.4	1.0 ± 0.1
Test pea	2.2	2.0	ND	1.4 ± 0.4	ND	4.4 ± 0.9[c]	ND	1.3 ± 0.2
Blue Buffalo	23.0	3.0	ND	1.0 ± 0.2	ND	1.9 ± 0.3[b]	0.4 ± 0.2	0.7 ± 0.1
Iams	17.7	2.4	ND	0.9 ± 0.2	1.9 ± 1.9	4.3 ± 0.6[a,c]	0.2 ± 0.1	1.3 ± 0.3
Purina	39.9	2.7	ND	0.9 ± 0.2	ND	3.8 ± 0.4[a,c]	0.2 ± 0.1	1.1 ± 0.1

Values shown as mean ± SEM, n=4 for plasma, urine and fecal samples. Feed samples are shown as averaged values from duplicate determinations of the same sample. Values in a column with superscripts without a common letter differ, $P < 0.05$; 1-way ANOVA with LSD post-hoc test. Columns without any superscripts showed no significant differences among diets in 1-way ANOVA.

[1]Not detectable (ND)

Table 3.9 shows the levels of ammonia and urea in dog feed as well as dog plasma, urine and fecal samples obtained after feeding each diet (two days for urine and six days for plasma and feces). Concentrations of ammonia were higher than urea in all feed samples, but all feeds were low for both compounds. The dog feed concentrations of ammonia ranged from 1.5-25.4 mg/kg, while urea ranged from 3.2-7.2 µg/kg (Table 3.9). Diets within the mid-range for crude protein content contained the lowest levels of both ammonia and urea in feed (Table 3.9), thus protein content did not appear to be the driver for ammonia or urea levels in feed. However, in plasma samples, when these same mid-range diets were fed to dogs, they resulted in significantly higher ammonia and significantly lower urea ($P<0.05$; Table 3.9). There did not appear to be a primary route of excretion for ammonia and urea in dogs, as concentrations were similar between urine and feces (Table 3.9). In urine, ammonia concentrations did not differ significantly with the different diets. In contrast, with urine urea, dogs fed the Purina diet had significantly lower urine urea than all other diets ($P<0.05$; Table 3.9). In canine fecal samples, urea levels did not differ significantly among the diets, while the diet from Orijen had significantly higher levels of fecal ammonia than the lower-priced, lower-protein containing dog feeds ($P>0.05$; Table 3.9).

Table 3.9 Ammonia and urea concentrations in dog feed as well as plasma, urine, and feces after being fed commercial diets or a lab diet (test pea) for two days (urine) and six days (plasma and feces). Diets are listed in decreasing level of crude protein inclusion.

	Feed		Plasma		Urine		Feces	
Diet	Ammonia (mg/kg)	Urea (µg/kg)	Ammonia (mg/l)	Urea (µg/l)	Ammonia (mg/l)	Urea (µg/l)	Ammonia (mg/kg)	Urea (µg/kg)
Orijen	19.5	7.22	$5.7 \pm 2.8^{a,c}$	$20.7 \pm 1.6^{a,c}$	59.5 ± 4.3	14.6 ± 1.7^{a}	89.8 ± 3.5^{b}	8.3 ± 0.6
Test pea	25.4	4.17	$7.7 \pm 1.6^{a,b,c}$	$16.7 \pm 1.8^{a,b}$	46.7 ± 7.5	17.7 ± 1.7^{a}	$61.9 \pm 10.7^{a,b}$	9.4 ± 0.4
Blue Buffalo	1.8	3.1	$11.7 \pm 1.8^{a,b}$	15.5 ± 1.1^{b}	58.0 ± 13.6	15.2 ± 3.5^{a}	76.0 ± 1.7^{a}	9.5 ± 0.3
Iams	5.3	3.78	14.0 ± 3.1^{b}	$18.1 \pm 1.7^{a,b}$	64.1 ± 4.8	14.7 ± 1.4^{a}	59.4 ± 6.7^{a}	10.9 ± 1.0
Purina	20.3	4.19	4.2 ± 2.0^{c}	23.6 ± 2.0^{c}	64.8 ± 22.8	5.9 ± 2.9^{b}	59.7 ± 4.4^{a}	10.2 ± 0.5

Values shown as mean ± SEM, plasma n=8, urine and feces n=4. Feed samples are shown as averaged values from duplicate determinations of the same sample. Values in a column with superscripts without a common letter differ, $P < 0.05$; 1-way ANOVA with LSD post-hoc test.

3.5.4 Cardiovascular changes and biomarkers of toxicity

After six days of feeding each diet to dogs, there were no statistically significant differences in cardiovascular endpoints ($P>0.05$; Table 3.10) including blood pressure, heart rate, stroke volume, cardiac output and flow-mediated dilation. C-reactive protein was not detectable in any of the plasma samples.

However, there were significant differences in methemoglobin and nitrotyrosine levels, as shown in Figure 3.1. Plasma methemoglobin was significantly higher in dogs fed the Blue Buffalo and Purina diets for six days compared to all other diets ($P<0.05$; Figure 3.1). Conversely, plasma nitrotyrosine was significantly higher in dogs fed diets Orijen compared to when dogs were fed Blue Buffalo or Purina diets ($P<0.05$; Figure 3.1). Despite significant changes, it is important to note that both methemoglobin and nitrotyrosine remained at subclinical levels for all tests, with methemoglobin not exceeding 1.5% and nitrotyrosine remaining below 2 μM.

Table 3.10 Echocardiography and blood pressure measurements in dogs after six days of feeding commercial diets or a lab made diet (test pea). Diets are listed in decreasing level of crude protein inclusion.

Diet	Systolic pressure (mmHg)	Diastolic pressure (mmHg)	Heart rate (bpm)	Stroke volume (ml/beat/kg)	Cardiac output ($l/kg^{-1}min^{-1}$)	Flow mediated dilation (%)
Orijen	144 ± 3.6	70 ± 2.3	67 ± 4.1	1.2 ± 0.1	10 ± 0.9	3.3 ± 0.6
Test pea	145 ± 5.9	73 ± 4.4	76 ± 5.7	1.1 ± 0.1	8 ± 0.9	3.6 ± 0.6
Blue Buffalo	137 ± 2.8	77 ± 2.2	71 ± 5.3	0.9 ± 0.1	8 ± 0.9	5.2 ± 0.8
Iams	137 ± 2.7	73 ± 2.2	71 ± 6.9	1.1 ± 0.1	9 ± 0.8	3.5 ± 0.4
Purina	134 ± 2.6	73 ± 2.2	67 ± 6.4	1.1 ± 0.04	11 ± 1.1	3.1 ± 0.7

Values shown as mean ± SEM, n=8. No significant differences were observed for any end-points, $P > 0.05$; 1-way ANOVA.

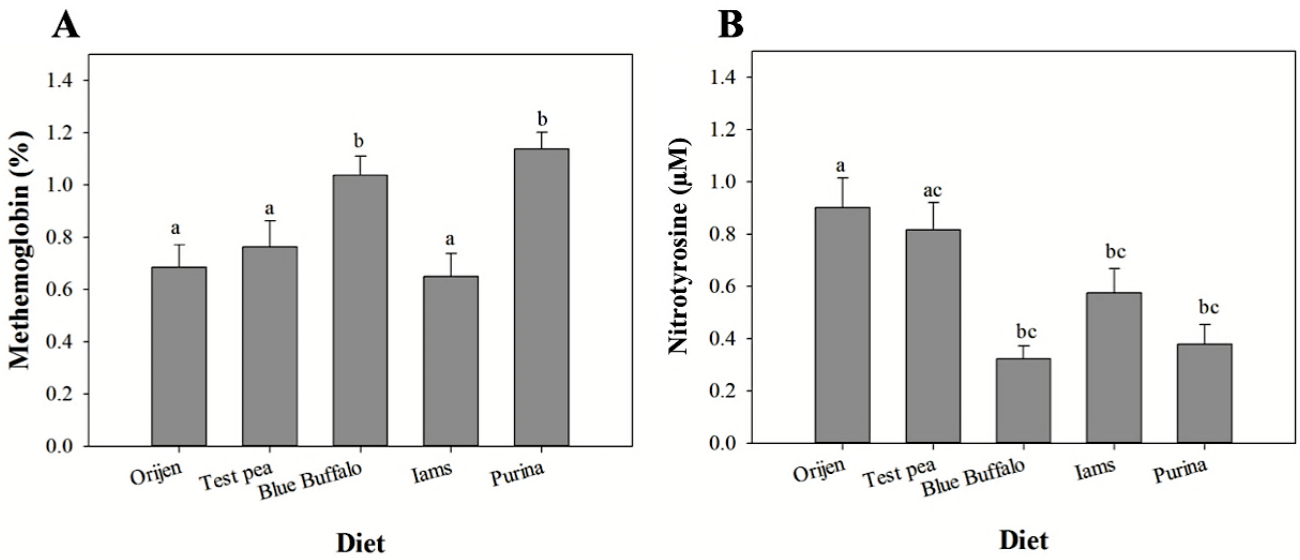


Figure 3.1 Biomarkers of toxicity in dogs after six days of feeding commercial diets or a lab made diet (test pea). Methemoglobin (A) and nitrotyrosine (B) analyzed in plasma samples of dogs fasted overnight. Values shown as mean ± SEM, n=8. Values with superscripts without a common letter differ, $P<0.05$; 1-way ANOVA with LSD post-hoc test. Diets are shown in order of decreasing crude protein inclusion from left to right.

3.5.5 Regressions

There were significant linear relationships between crude protein percentage in the diets tested and several endpoints measured in the dog study, as illustrated in Figure 3.2. There was a positive relationship between crude protein in dog diets and crude fat ($P=0.02$, $R^2>0.6$) as well as a weak relationship between price and fecal nitrite ($P=0.08$, $R^2>0.6$). Figure 3.2 also shows the weak negative relationship between crude protein and either urine nitrate ($P=0.07$, $R^2>0.6$) or plasma ammonia ($P=0.06$, $R^2>0.6$). Finally, a strong positive relationship was found between dietary ammonia concentration and protein digestibility in dogs ($P=0.01$, $R^2>0.6$), as shown in Figure 3.2.

Results of the cat study showed some similar relationships between crude protein and endpoints measured in this study (Figure 3.3). There was a very weak positive relationship between crude protein and price of cat foods ($P=0.21$, $R^2>0.6$). Price is one of foremost factors pet owners consider when deciding on a brand. For this reason, relationship between cat food price and crude protein was still included, despite the results being non-significant. There was also a positive relationship between crude protein with crude fat ($P=0.01$, $R^2>0.6$). Furthermore, there were significant relationships between crude protein in the cat diets with either dietary nitrite or urine nitrate. There was a negative relationship between diet crude protein and dietary nitrite in cat foods ($P=0.04$, $R^2>0.6$) as well as a positive relationship between dietary crude protein and urine nitrate with cat foods ($P=0.02$, $R^2>0.6$).

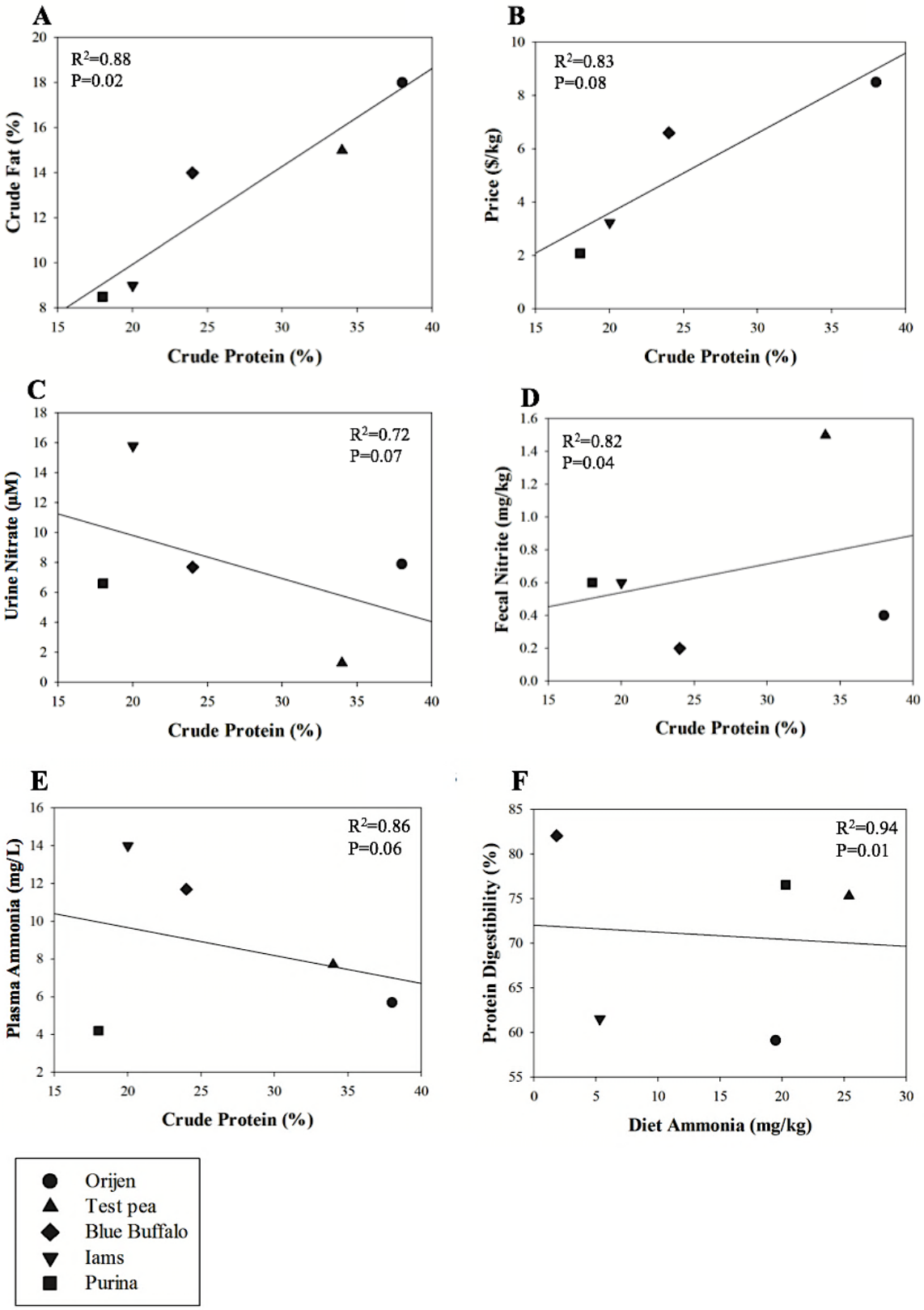


Figure 3.2 . Simple linear regression showing relationship between crude protein and other significant endpoints in dogs after six days of feeding commercial diets or a lab made diet (test

pea). (A) Positive relationship between crude protein and crude fat. (B) Weak positive relationship between crude protein and price. (C) Weak negative relationship between crude protein and urine nitrate concentration. (D) Positive relationship between crude protein and fecal nitrite concentration. (E) Weak negative relationship between crude protein and plasma ammonia concentration. (F) Negative relationship between diet ammonia and protein digestibility. Regression lines shown for relationships where $R^2>0.6$.

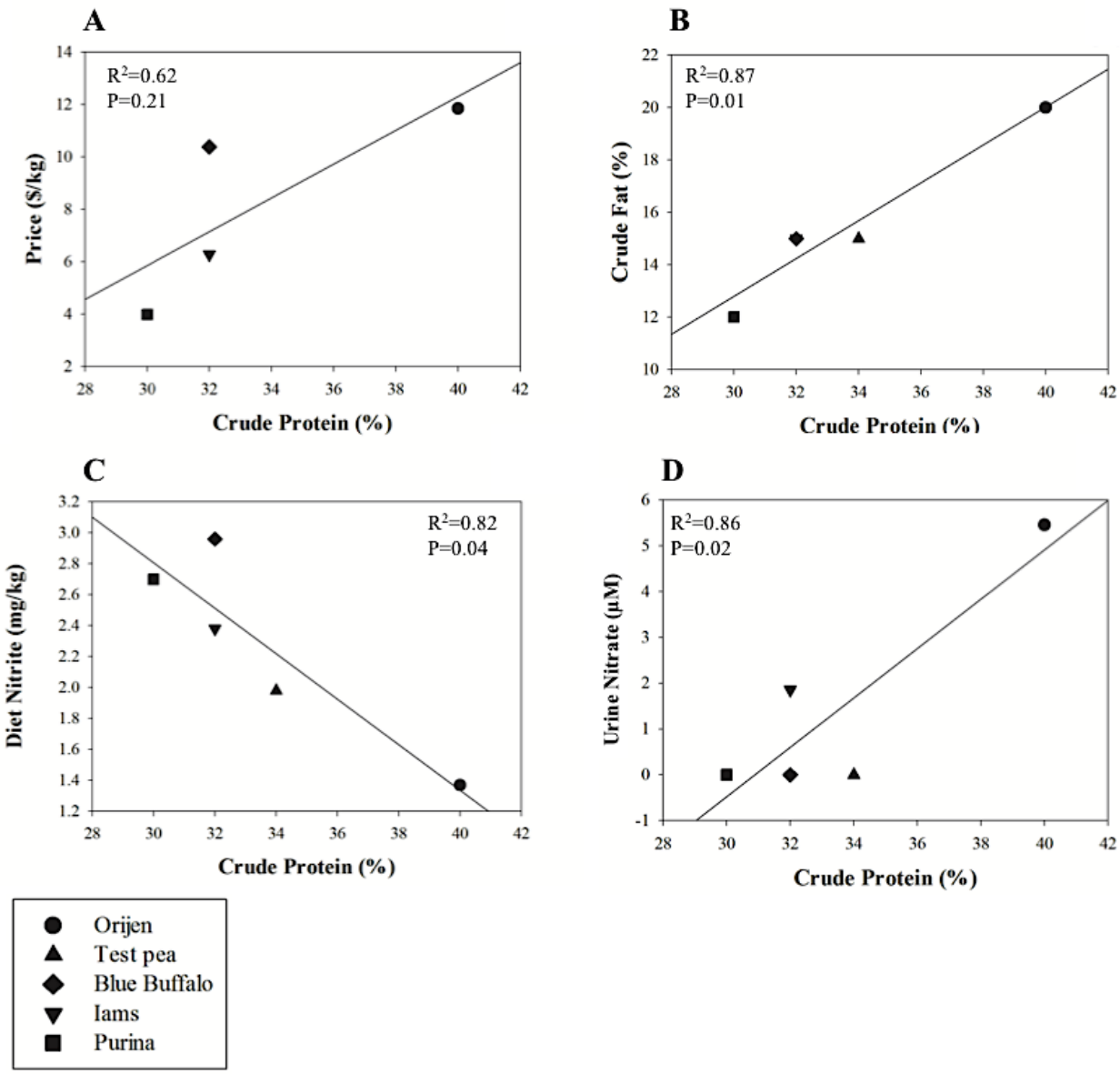


Figure 3.3 Simple linear regression showing relationship between crude protein and other significant end points in cats after six days of feeding commercial diets or a lab made diet (test pea). (A) Weak positive relationship between crude protein and price. (B) Positive relationship between crude protein and crude fat. (C) Negative relationship between crude protein and diet nitrite concentration. (D) Positive relationship between crude protein and urine nitrate concentration. Regression lines shown for significant relationship, $R^2>0.6$.

3.6 Discussion

The present study examined differences in protein quality and utilization of commercial pet food, in dogs and cats. The major findings of the descriptive analysis of the commercial diets indicated that over a range of price points, protein inclusion differed. As the most expensive ingredient, animal protein inclusion reflected price. Diets at a higher price point contained a greater crude protein content, as well as a greater variety of ingredients and more expensive sources of animal and plant protein (Pichon et al. 1983). Protein content also increased with fat content and caloric density. Therefore, if daily food portions during feeding are not corrected for caloric density by the pet owner, the higher energy and fat content of more expensive feeds could contribute weight gain and diseases associated with obesity. A 2010 study by German et al. stated that active dogs require greater maintenance energy requirements (MER). These dogs would benefit from a higher protein, higher calorie diet, whereas the average companion canine with a relatively sedentary life would instead benefit from a high fibre diet. In the German et al study, the dogs who received moderate to low exercise showed weight loss on a high fibre diet. With 34-59% of dogs entering veterinary clinics being overweight, high protein, high calorie diets like the high priced diets in the present study should be avoided, unless properly portioned to account for the MER of the dogs to which they are being fed (Switonski and Mankowska 2013). Thus, owners buy more expensive diets thinking they are healthier may in fact be feeding these diets long-term. This inadvertently promotes weight gain and diseases associated with it like diabetes mellitus, cardiovascular disease and dystocia, among others (Gossellin et al. 2007).

All diets met and exceeded the AAFCO minimum crude protein inclusion of 18% for adult maintenance in dogs and 26% in cats (AAFCO 2013). With some commercial diets containing up to 38% crude protein in dog diets and 40% crude protein in cats, certain diets are actually over supplementing with protein to the point where the animals cannot absorb and incorporate all of the included protein into tissues. Nitrogen retention estimates the bioavailability of nitrogen in the diet and how much of that nitrogen is absorbed and utilized by the animal (Ammerman et al. 1995). Ultimately, the excess protein in the high protein diets used in this study would be metabolized for energy or stored as fat, with nitrogen from amino acids wasted through excretion (Beynen et al. 2002). This is supported by the fact that, while not statistically significant, nitrogen retention was lower in the highest priced diets in both species. However, the high priced diets still maintained a high protein digestibility, indicating greater

protein quality and bioavailability in these diets. The high priced diets contained a wider variety of animal protein products, meanwhile the lower protein diets included one or two animal protein sources, with supplemental plant sources. The high priced diets also contained fish protein sources as opposed to exclusively chicken or pork meal. A 2005 study by Dust et al. examined the digestibility of different protein sources used in pet food. It was determined that of the animal proteins tested, fish proteins were more digestible in cannulated dogs than beef, pork or chicken. The study noted that while digestibility values were high for all protein sources, protein ingredients containing more fibre, bone, collagen or connective tissue had lower digestibility. Cats are considered to be obligate carnivore species, with higher protein requirements than dogs (Bradshaw 2006). Similar to dogs, with the high priced feline diets containing greater animal protein, it would allow for a more efficient protein digestibility than the low priced diets with greater plant protein inclusion (Funaba et al. 2002). A review by Rogers and Morris 1982 examined protein utilization in cats compared to rats and dogs. The results of the study determined that when fed low protein diets, cats had more nitrogen loss than either dogs or rats. It was stated that this could be the result of cat livers not being able to adjust to the activity of nitrogen catalytic enzymes when there is a shift to a lower level of dietary protein. The review also stated that cats are metabolically programmed to digest only medium to high protein diets and are not as enzymatically efficient when switched to a low protein diet. With the diets in the current study all exceeding the minimum AAFCO crude protein requirement for cats, this is consistent with the observed high nitrogen retention. In contrast, we questioned the accuracy of the current study's protein digestibility in the cats (zero digestibility was almost certainly inaccurate). In comparison, a similar study assessing wet and dry cat food determined crude protein digestibility to be low, ranging from 67-87% (Kendall et al.1982). In the current study, most protein digestibility values in cats fell below this range. Thus, when considered in conjunction with the high nitrogen retention, it is almost certain that cat protein digestibility was inaccurate in this current study. Commercial cat food is often coated in extra fat and palatants (Lin et al. 2019) to which the chromium may have adhered, resulting in the marker not being evenly distributed through all of the feed and subsequent inaccurate estimations of digestibility.

The concentration of toxic nitrogenous compounds, including nitrate, nitrite, urea and ammonia, in feed and biological samples were all low and levels of nitrite did not exceed the maximum FDA limit of 20 ppm in the feed (FDA 2018). The high priced diets from Orijen did

contain the highest concentrations of nitrate, but it is unlikely that this manufacturer was trying to boost the total apparent crude protein with non-nitrogen protein. The high nitrate content is likely due to the manufacturer inclusion of high nitrate-containing plant sources, such as peas and green, leafy vegetables, including kale and spinach (Bondonno et al. 2014). A study by Lehman 1958, tested different inclusions of nitrate in pet products. It was determined that less than 2% inclusion of dietary nitrate per day was the no observable adverse effects level in dogs. However, long-term exposure of moderate dietary nitrate could potentially lead to lipid peroxidation and oxidative stress as a result of nitrate cycling and the production of reactive oxygen species (Brunning-Fan et al. 1993). This coincides with the nitrotyrosine findings of this study, where the diet containing the highest protein and nitrate, also produced the highest plasma nitrotyrosine concentrations in dogs after six days of feeding. Nitrotyrosine is a biomarker of oxidative stress in the body. Higher levels of nitrotyrosine are indicative of oxidative stress caused by cycling of nitrogenous compounds like nitrate, nitrite and nitric oxide (Mohiuddin et al. 2006). In contrast, in the current study we cannot comment on cats since this was determined only in dogs, we observed lower methemoglobin levels seen in the dogs fed the diets highest in nitrate. Previous studies have shown that at subclinical levels of nitrate, there may be a reversal of methemoglobin formation, as a result increased expression or activity of the methemoglobin reducing enzyme, methemoglobin reductase (Duncan et al. 1997). While methemoglobin reductase is not well studied in mammalian species, a study by Jensen and Nielsen (2018) examined methemoglobinemia recovery in rainbow trout. It was determined that a positive recovery of methemoglobin formation was the result of methemoglobin reductase stimulation as a response to low oxygen saturation.

This study also indicated that as the level of crude protein increases in commercial dog diets, so does the level of dog fecal nitrite. This may indicate that some of the dietary nitrate or other nitrogenous compounds in the diet was converted into nitrite in the gut and excreted through the feces. It could also be that a portion of the dietary nitrite was not absorbed and directly excreted. Nitrate is often found at greater concentrations in pet food than nitrite. Nitrate is commonly added as plants sources, whereas nitrite is usually included as a meat preservative (Bahadoran et al 2016). Upon ingestion, much of the nitrate is converted into nitrite as a result of a combination of low pH, microbial population and salivary enzymes in the mouth (Sukuroglu et al. 2015). As the nitrite moves through the gastrointestinal system, it can undergo cycling and be

converted back into nitrate, converted into other nitrogenous compounds or be excreted as nitrite (Fritsch et al. 1985). In contrast, there was a negative relationship observed between crude protein and urine nitrate in dogs. This could mean that the sources of nitrate in the high protein diets was more accessible by the animal and more readily converted into nitrite or other nitrogenous compounds in the gut. A 2008 study by Van Velzen et al. examined the oral bioavailability of nitrate in human foods. It was determined that 80-85% of dietary nitrate came from fruit and vegetable sources. Of those sources, beet root and leafy vegetables like spinach had the greatest nitrate bioavailability. This supports the findings of the present study, as the high priced diets contained spinach, kale and beet root and were also associated with the highest fecal nitrite, while the lower priced diets did neither. In contrast, urinary nitrate showed a positive relationship with dietary crude protein in cats. This indicates a species difference between cats and dogs when comparing crude protein content and urinary excretion of nitrate. A 2004 study by Zentec and Shulz examined urinary composition of cats fed diets increasing in protein. It was determined that when cats were fed diets increasing in protein, there was also a subsequent increase in urine nitrogen and ammonia. Since ammonia is a reduced product of dietary nitrate (Tiso and Schechter 2015), this could be an indicator that a portion of the urinary nitrogen increase observed by Zentec and Shulz could be from nitrate. This supports the findings of the present study where urinary nitrate concentration increased with crude protein content in cats.

Ammonia and urea were at low levels in the dog diets and biological samples. However, ammonia increased in concentration between ingestion and excretion, indicated by higher levels in the urine and feces than in the feed and plasma. Ammonia in the urine and feces may arise from a small extent from ammonia added in the diet, but the majority of urinary and fecal ammonia more likely arose when other nitrogenous compounds, including amino acids, from the diet were converted into ammonia in the liver. Ammonia is produced as a by-product of the metabolic process where amino acids are transaminated to pyruvate for energy (Lowenstein 1972). A review by Huizenga et al. 1996, also stated that a combination of low gastrointestinal pH and presence of microorganisms promoted the deamination of α-amino acids to ammonia in the large and small bowl of dogs. Even compounds like dietary nitrate and nitrite can be reduced into ammonia under a low pH (Becer et al. 2010). Additionally, during the urea cycle, trace amounts of ammonia in food are usually converted by mammals into a non-toxic form (Kung et al. 2000). Concentrations of urea were highest in plasma and urine, labeling urine as the primary

route of excretion for urea in dogs (Bankir and Yang. 2012). Results of the present study also showed that concentrations of ammonia were higher than urea in all samples. Higher levels of ammonia in the urine and feces of all diets indicate that the majority of ammonia ingested and produced during metabolism and digestion is not converted into urea during urea cycling. Ammonia nitrogen only makes up approximately 10% of urea nitrogen excretion, with approximately 50% of ammonia produced during metabolism being excreted directly in the urine (Weiner et al. 2015). It is unlikely that the ammonia found in the commercial diets was added as non-protein nitrogen to boost apparent protein content and instead results fit with the scenario that diets with high animal protein used ammonia as a preservative. Anhydrous ammonia is specifically used to reduce the incidence of *Escherichia coli* in beef products after processing. Similarly, ammonium hydroxide is used in a variety of non-meat products to reduce incidence of pathogenic microbial species (Tajkarimi et al. 2008). Thus, it is most like the feed ingredient manufacturers, not the pet food companies themselves that have added ammonia for this purpose. The positive relationship observed between dietary ammonia and protein digestibility in the current study further supports this hypothesis. It is unlikely that the dietary ammonia improved the protein digestibility, but instead the more digestible animal proteins in higher priced dog foods had more ammonia added as a preservative.

There were no differences in any of the cardiovascular parameters after feeding any of the diets for six days and all values were within normal ranges for dogs (Hopper 2009). While we had hypothesized based on largely human literature that high dietary nitrate would enhance flow-mediated dilation and reduce blood pressure (Jonvik et al. 2016), dietary nitrate appears to lack the same vasodilatory potential in dogs as it does in humans. Companion animals and humans share many of the same cardiovascular traits, which means that they are susceptible to many of the same cardiovascular illnesses (Mubanga et al. 2017). However, there are certain cardiovascular diseases that can affect certain sizes and breeds more than others. Unlike in humans, hypertension is less prevalent in dogs. Approximately 10% of dogs develop hypertension, with most of them being senior animals (Remillard et al. 1991). Canine hypertension is diagnosed when systolic pressure exceeds 160 mmHg. Secondary hypertension is much more prevalent in dogs than primary hypertension, where there is usually an underlying disease, like renal failure, that causes high blood pressure (Serres et al. 2006). Obesity is another major contributor to hypertension in dogs, which can be linked to nutrition (Hall et al. 2000).

The beagles used in the present study were in healthy condition and middle aged. Changes in blood pressure as a result of nitrate and nitrite exposure may not have been as evident in healthy animals. Also, the levels of nitrate and nitrite used in the diets tested may not have been high enough, fully bioavailable or fed for long enough to elicit an effect. A 2016 study by Jonvik et al. examined the use of dietary nitrate in the form of spinach, beet pulp and sodium nitrate, as a vasodilatory agent in humans. The researchers were able to observe changes in blood pressure and flow mediated dilation where the dose of dietary nitrate was 800 mg/kg/day, plasma nitrate ranged from 61-69 μM and plasma nitrite ranged from 115-155 μM. These concentrations in both the diet and plasma are much greater than what was found in the present study in dogs. Ultimately, the concentrations of nitrate and nitrite in the commercial canine diets were high enough to influence vascular distensibility or cause changes in cardiac function.

3.7 Conclusions

These findings indicate that it is not necessary for pet owners to spend money on high priced diets, where protein is concerned. It may be most beneficial for pet owners to invest in moderately priced diets in order to avoid health problems with weight gain and prolonged exposure to inflammatory proteins, as seen with the high protein, high priced diets. However, while there were differences in detection of non-protein nitrogen in the commercial diets, they were not in toxic concentrations. Protein handling in both cats and dogs was similar among all commercial diets. Furthermore, adult maintenance commercial diets do not possess an observable therapeutic cardiovascular potential in dogs. The concentrations of nitrate and nitrite were likely not at high enough concentrations or fully bioavailable in diet ingredients to produce a vascular effect, but results should be confirmed in a longer-term feeding study.

4.0 THERAPEUTIC POTENTIAL VERSUS TOXICITY OF NITRATE AND NITRITE ON THE CANINE CARDIOVASCULAR SYSTEM

4.1 Preface

Chapter 4 is the second of two studies included in this thesis on nitrate and nitrite inclusion in pet food. Unlike the previous study, Chapter 4 uses dogs as the single species of analysis. This chapter explores the potential therapeutic qualities of nitrate and nitrite used as a vasodilatory agent in pet food, in order to improve vascular distensibility and vascular endothelial function. This study also explores the potential relative toxicity associated with nitrate doped pet food.

This chapter will be submitted for publication in the *Journal of Animal Physiology and Animal Nutrition (Japan).* Geiger (responsible for 100% of all animal work, all biochemical tests except those where samples were sent to a contract lab for proximate analyses, diet formulations, all data analyses and all writing), Weber (supervised project, helped with study design and editing), Drew (assisted with diet formulation).

4.2 Abstract

Nitrate and nitrite are present in canine feeds due to incorporation of plant materials high in these compounds, as well as use as a preservative for protein ingredients. These nitrogenous compounds have the potential to induce toxic effects such as methemoglobinemia, lipid peroxidation and disruptions in cardiovascular function. Conversely, nitrate and nitrite may improve vascular function and reduce hypertension due to conversion into nitric oxide. To examine this relationship, five canine diets were formulated to which plant based or pure nitrate or nitrite was included at <20 ppm. Eight dogs were randomly assigned and fed each diet for six days (n=8 dogs/diet). At the end of each trial, plasma was collected for nitrate, nitrite nitrotyrosine and methemoglobin analysis. In addition, the cardiovascular health of dogs fed the five diets was analyzed using echocardiography and flow mediated dilation. Plasma and dietary nitrate was high in all of the diets, ranging from 99-115 mg/kg in the diet and 27-65 μM in the plasma. A further nitrate/nitrite analysis of the ingredients revealed that the chicken meal used as

the primary protein ingredient was very high in nitrate, containing 115 mg/kg nitrate. A proximate analysis revealed that crude protein of the diets was higher than the 18% that was originally formulated, possibly as a result of the excess nitrate. There were cardiovascular differences between dietary nitrate and nitrite. With increasing dietary nitrate, there was a positive relationship with heart rate (R^2=0.69) and stroke volume (R^2=0.69), with no influence of flow mediated dilation. While dietary nitrite had a negative relationship with heart rate (R^2=0.67) and a positive relationship with flow mediated dilation (R^2=0.75). Ultimately, results suggest that incorporating <20ppm of dietary nitrite in canine diets may have the potential to improve vascular distensibility, without having a toxic effect. Meanwhile, nitrate incorporated into canine diets may have the potential to boost apparent protein content and cause negative effects on the cardiovascular system, with increases in heart rate and stroke volume.

4.3 Introduction

Nitrate and nitrite are nitrogenous compounds with both potential therapeutic and toxic qualities. When incorporated as a dietary source, nitrate and nitrite have the potential to produce a vasodilatory effect through conversion to nitric oxide in circulation (DeMartino et al. 2019). As an endogenous vasodilator, nitric oxide improves vascular distensibility by stimulating the relaxation of vascular smooth muscle which in turn reduces both blood pressure and cardiac afterload (Daiber et al. 2019).

Nitrate and nitrite can be incorporated into canine diets either as an inorganic protein preservative or from plant sources like spinach, kale, beet root, and peas (ESFA 2008). Nitrate in excess, as a non-protein nitrogen source, has the ability to boost the apparent crude protein content of pet food diets (Li et al. 2015). However, when added in excess, nitrate and nitrite can cause lipid peroxidation, methemoglobin formation and even death at the highest concentrations (Carriker et al. 2018). The FDA and AAFCO have set nutritional limits in order to avoid this type of toxicity and ensure proper nutritional maintenance in pet foods (AAFCO 2013, FDA 2018). Macronutrient content, including minimum crude protein, is regulated by AAFCO in order to ensure adequate nutritional quality of commercial pet foods (AAFCO 2013). There is no limit set by the FDA regarding nitrate in pet food, while the maximum nitrite concentration in pet food cannot exceed 20 ppm (FDA 2018).

The purpose of this study was to assess the therapeutic and toxic effects dietary nitrate and nitrite in dogs. It was hypothesized that with added nitrate and nitrite, there would be improved vascular distensibility and decreased blood pressure without a change in cardiac function, while also showing increased oxidative stress and methemoglobin levels. In order to investigate this hypothesis, a six day feeding study using healthy, adult research beagles in a randomized cross-over design tested 5 different diets (specify these diets here). At the end of each six day feeding period, cardiovascular responses were determined using echocardiography, flow-mediated dilation (tests endothelium-dependent relaxation) and high-definition oscillometry (indirect blood pressure). These measurements were then related to dietary and plasma nitrate or nitrite levels plus blood methemoglobin and plasma nitrotyrosine (indicator of oxidative stress) levels.

4.4 Materials and methods

4.4.1 Animals

Eight adult beagle dogs (four spayed females and four neutered males) of 5±0.5 years of age at the beginning of the trial were originally obtained from a certified scientific breeder (Marshall Farms, NY) and housed at the animal care facility at the Western College of Veterinary Medicine (Saskatoon, SK). Dogs were kept in individual kennels for feeding and overnight, but had open kennels to allow socializing with each other as well as access to outdoor runs during the day. When not on trial, dogs were fed a standard commercial adult maintenance pet food diet (Hills Pet Nutrition Inc, Topeka, Kansas, USA). The weight of food fed per animal per day varied was adjusted individually to maintain ideal body condition score (4-6 on 9-point Purina body condition scale) and was based on data obtained over several years for each animal. Dogs were clinically healthy prior to and throughout the study (based on general appearance and behaviour). All procedures and handling involving dogs were completed according to protocol approved by the University of Saskatchewan's Animal Research Ethics Board according to guidelines established by the Canadian Council on Animal Care.

4.4.2 Diet formulation

Five canine diets were formulated using the Concept 4 Creative software (Creative Formulation Concepts, LLC, Annapolis, MD, USA). A control diet was initially formulated, then this base diet re-formulated with sodium nitrate, sodium nitrite, beet pulp or whole ground peas added as the supplementary nitrate/nitrite source. Sodium nitrate and nitrite were each added to corresponding test diets at 0.002% inclusion to reflect the FDA 20 mg/kg maximum limit of nitrite (FDA 2018). Beet pulp was added to the beet diet at 7.5% inclusion, while whole ground field peas were added to the pea diet at 20% inclusion. Both the beet pulp and peas were added at inclusion rates that are similar to that currently used in the pet food industry in North America for each ingredient (Fayhey et al. 1992). To create diets, ingredients for specific diets were mixed using a commercial grade Hobart mixer (Hobart Food Equipment Canada, Toronto, ON, Canada), then municipal tap water added until desired consistency (approximately 33% (w/w)) and cold-pelleted by feeding through a round die attached to a Chef's Choice continuous meat grinder (Edgecraft Corporation, Avondale, PA, USA). Feed was dried at 65°C for two days or until dry. All diets were formulated to meet the minimum AAFCO nutrient requirements for dogs (AAFCO 2013), with crude protein set at 18%., crude fat set at 6%, crude fibre set at 3%, ash set at 4% and metabolizable energy set at 3120 kcal/kg in all diets. All diet ingredients and inclusion rates are listed in Table 4.1.

Table 4.1 Formulation and inclusion rates of ingredients for five canine test diets.

	Ingredient inclusion rate in diets (%)				
Ingredients	Pea	Nitrate	Control	Beet	Nitrite
Chicken meal	46	42	42	37	42
Fish meal (menhaden)	21	20	20	20	20
Brown rice flour	0	25	25	25	25
Wole ground peas	20	0	0	0	0
Dried beet pulp	0	0	0	7.5	0
Wheat flour	5	5	5	5	5
Canola oil	2	2	2	2	2
Solka flok	2	2	2	2	2
Celite	1	1	1	1	1
Potassium chloride	0.9	0.9	0.9	0.9	0.9
Dicalcium phosphate	0.5	0.5	0.5	0.5	0.5
Calcium carbonate	0.5	0.5	0.5	0.5	0.5
Salt	0.5	0.5	0.5	0.5	0.5
Choline chloride	0.4	0.4	0.4	0.4	0.44
Vitamin premix	0.1	0.1	0.1	0.1	0.1
Mineral premix	0.1	0.1	0.1	0.1	0.1
Sodium nitrate	0	0.02	0	0	0
Sodium nitrite	0	0	0	0	0.02

4.4.3 Feeding trials

Feed portions were calculated based on body condition score and body weight, with reference to formulated digestible energy per weight to produce isocaloric portions during testing. All eight dogs were fed the five different diets in a linear design, with each diet being fed sequentially in the same order (control, pea, beet, nitrate, nitrite). Diets were fed to beagles (n=8) for six days on each diet in their home kennels, then fasted overnight and followed by ultrasound testing the morning of day seven. Prior to fasting, fecal samples were collected on day six for determination of macronutrient digestibility. Celite marker (acid-insoluble ash), crude protein, fat and fibre were determined in all diet and fecal samples by a commercial feed analysis lab (Central Testing Laboratories, Winnipeg, MB, Canada). Methods used by this company were industry standard. Acid insoluble ash was measured by boiling sample in hydrochloric acid, after which insoluble ash was weighed. Crude protein was measured using the Kjeldahl method, where sulfuric acid is used to degrade the sample and free nitrogen. Crude fat was measured Soxhlet method, where fat is extracted using solvent. Crude fibre is a measure of the quantity of indigestible cellulose, pentosans, and lignin remaining after solvent extraction followed by digestion with dilute acid and alkali. These values were used to calculate macronutrient digestibilities.

Nutrient digestibility was calculated as:

Equation 4.1

$$\text{Nutrient digestibility (\%)} = 1 - \left[\frac{\%nutrient\ in\ feces \times \%celite\ in\ feed}{\%nutrient\ in\ feed \times \%celite\ in\ feces}\right]$$

4.4.4 Cardiovascular ultrasound

All dogs were previously acclimated to all blood collection and ultrasound procedures by providing positive attention during testing and treats after all procedures were done. Thus, the dogs were highly cooperative and we were able to examine the dogs without stress or any sedation for these procedures. Prior to ultrasound, dogs were weighed and blood pressure was taken using a high definition canine oscillometer (S + B medVET GmbH, Babenhausen, Germany). Endpoints of flow mediated dilation included brachial artery diameter during baseline, during inflation of a blood pressure cuff placed distal to the brachial artery and at the

time of peak dilation (30 seconds) after cuff release previously determined in our group in dogs (Raitkatari et al. 2000; Adolphe et al. 2012). Echocardiography endpoints to assess cardiac function included, heart rate, stroke volume and cardiac output (Otto et al. 2019; Adolphe et al., 2012). Flow mediated dilation and echocardiography were measured using a SonoSite Edge II ultrasound (Fujifilm SonoSite inc., Bothell, USA), with detection using the P10x transducer (8-4 Hz) to detect cardiac endpoints and the L38xi (10-5 Hz) transducer to measure flow-mediated dilation. After ultrasound was conducted, a 3.0 ml aliquot of plasma was obtained for use in nitrate, nitrite, and nitrotyrosine assays. Also, a 1.0 ml aliquot of blood gases was collected for methemoglobin analysis using a blood gas electrolyte analyzer (Shinova Medical Co., Shanghai, China).

4.4.5 Nitrate, nitrite and biomarker analysis

Nitrate and nitrite concentrations in each diet, major feed ingredients and plasma were determined using a commercially available nitrite/nitrate assay kit based on the greiss colour reaction (R&D Systems, Bio-Techne Corporation, Minneapolis, MN, USA). Plasma was analyzed directly in the assay, while feed and solid ingredient nitrate and nitrite was extracted into solution. Solid feed and ingredient samples were ground and diluted using a 10X dilution in reagent-grade water. Diluted samples were heated at 60°C for three hours to extract nitrogenous compounds. All samples were filtered using a 10 kDa cut-off filter to reduce protein interference in the colorimetric assay. Nitrite was measured directly from the assay and nitrate was calculated based on subtracting nitrite from the total nitrate/nitrite detected. Nitrotyrosine, as a biomarker for oxidative stress, was analyzed in plasma samples using a commercially available nitrotyrosine ELISA assay (Hycult Biotech, Uden, The Netherlands).

4.4.6 Statistical analysis

All data was tested for parametric assumptions: a Levene's test was used to test for homogeneity of variance and a KS-test was used to test if data was normally distributed. All data met parametric assumptions. Data was analyzed using a 1-way ANOVA, with a Fisher's LSD post hoc pair-wise comparisons, with α set at 0.05. Linear regressions were used to determine the relationships between nitrate/nitrite concentrations and endpoints measured, with relationships deemed significant at $R^2>0.6$. Data are shown as mean±SEM. All data analysis was

performed using SPSS statistics version 25 (SPSS Chicago, Il, USA, IBM), using linear mixed models.

4.5 Results

All diets were consumed by all of the dogs, with no signs of food refusal. All dogs did however have diarrhea after being fed all five diets. Results are arranged in Tables and Figures in decreasing detected nitrate concentration.

4.5.1 Proximate analysis

The most notable findings of the proximate analysis of the diets revealed that crude protein was not detected at the formulated 18%, but instead appeared to range from 45-55%. Crude fat ranged from 8.8-9.8%, crude fibre ranged from 0.3-1.6%, ash ranged from 15-17% and metabolizable energy ranged from 3169-3344 kcal/kg (Table 4.2). All nutrient requirements met and/or exceeded the minimum AAFCO limits (AAFCO 2013).

Table 4.2 Proximate analysis of canine test diets.

Diet	Crude Protein (%)	Crude fat (%)	Crude fibre (%)	Ash (%)	Metabolizable energy (kcal/kg)
Pea	50.3	9.8	0.6	17.4	3169.7
Nitrate	49.8	9.3	0.3	15.6	3335.5
Control	49.1	9.4	0.6	15.4	3344.7
Beet	45.1	8.8	1.4	15.0	3270.7
Nitrite	55.3	9.4	1.6	16.2	3289.6

4.5.2 Nitrate and nitrite concentrations in ingredients, diets and dog plasma after feeding test diets for six days

To better characterize the sources of nitrate and nitrite in the test diets, all major feed ingredients used in the current study were analyzed. The nitrate and nitrite analysis of the feed ingredients showed that the chicken meal contained the greatest excess of nitrate, at 115.4 mg/kg nitrate (Table 4.3). Celite and fish meal contained the next highest concentrations of nitrate at 6.6 mg/kg and 1.5 mg/kg, respectively. All other major ingredients contained no detectable traces of nitrate. While most feed ingredients contained no detectable nitrite, whole ground peas contained the highest levels of nitrite, with 2.6 mg/kg nitrite, but this did not exceed FDA maximum pet food levels of 20 ppm (Table 4.3).

Table 4.4 shows the concentrations of nitrate and nitrite in the whole diets and plasma from dogs after six days of feeding each diet. Dietary nitrate ranged from 100-116 mg/kg, with the pea diet containing the highest concentrations of nitrate, while the nitrite diet contained the lowest concentrations of nitrate. Dietary nitrite ranged from 0-3 mg/kg (Table 4.4). Plasma nitrate ranged from 28-65 μM, with the pea and nitrite diets contained the only detectable traces of nitrite. Meanwhile, plasma nitrate was significantly highest in dogs fed the nitrate diet ($P<0.05$). Plasma nitrite ranged from 0-1.3 mg/kg but did not differ significantly among diets ($P>0.05$).

4.5.3 Protein and fat digestibility

As illustrated in Table 4.5, protein digestibility in the diets ranged from 75-81%, while fat digestibility ranged from 94-98%. Apparent protein digestibility differed significantly among the diets (1-way ANOVA; $P<0.05$; Table 4.5). While the pea and nitrite diet did not differ significantly from each other, the pea diet had significantly lower protein digestibility than the control, beet and nitrate diets. Fat digestibility also differed significantly among the diets ($P<0.05$; Table 4.5), with the pea diet having significantly lower fat digestibility than all other diets.

Table 4.3 Nitrate and nitrite concentrations in major ingredients used in canine test diets.

Ingredient	Nitrate (mg/kg)	Nitrite (mg/kg)
Chicken meal	115.4	ND
Celite	6.6	ND
Fish meal	1.5	ND
Peas	ND[1]	2.6
Beet pulp	ND	ND
Brown rice flour	ND	ND
Wheat flour	ND	ND
Solka floc	ND	ND

[1]Not detectable (ND)

Table 4.4 Nitrate and nitrite concentrations measured in canine plasma and feed samples after being fed test diets.

	Feed (mg/kg)		Plasma (μM)	
Diet	Nitrate	Nitrite	Nitrate	Nitrite
Pea	115.7	0.3	27.6 ± 8.0[a]	ND
Nitrate	114.7	ND[1]	65.6 ± 6.4[b]	ND
Control	113.0	ND	39.7 ± 8.9[a]	ND
Beet	103.2	ND	33.3 ± 6.0[a]	1.3 ± 1.3
Nitrite	99.6	3.0	34.8 ± 6.6[a]	ND

Values shown as mean ± SEM, n=8. Values in a column with superscripts without a common letter differ, $P < 0.05$; 1-way ANOVA with LSD post-hoc test. Diets are shown in order of decreasing nitrate level.
[1]Not detectable (ND)

Table 4.5 Protein and fat digestibility of canine test diets.

Diet	Protein digestibility (%)	Fat digestibility (%)
Pea	75.3 ± 1.9^{b}	94.7 ± 1.0^{b}
Nitrate	80.8 ± 1.1^{a}	97.7 ± 0.2^{a}
Control	81.0 ± 1.0^{a}	97.5 ± 0.3^{a}
Beet	78.9 ± 0.9^{a}	97.2 ± 0.3^{a}
Nitrite	$78.6 \pm 0.8^{a\ b}$	97.9 ± 0.2^{a}

Values shown as mean ± SEM, n=8. Values in a column with superscripts without a common letter differ, $P < 0.05$; 1-way ANOVA with LSD post-hoc test. Diets are shown in order of decreasing nitrate level.

4.5.4 Cardiovascular changes and biomarkers of toxicity

After 6 days of feeding different diets to dogs, there were no statistically significant differences in any cardiovascular endpoints among any of the diets ($P>0.05$; Table 4.6). In contrast, there were significant differences in methemoglobin and nitrotyrosine levels, as shown in Figure 4.1. Methemoglobin levels in the dogs fed the beet, nitrate and nitrite diets were significantly lower than the levels in the control diet ($P<0.05$; Figure 4.1). In contrast, after 6 days of feeding dogs the control diet, plasma nitrotyrosine was significantly lower than when dogs were fed the pea, nitrate or nitrite diets ($P<0.05$).

Table 4.6 Blood pressure, heart rate, echocardiography and flow-mediated dilation in dogs after six days of feeding test diets.

Diet	Systolic pressure (mmHg)	Diastolic pressure (mmHg)	Heart rate (bpm)	Stroke volume (ml/beat/kg)	Cardiac output ($l/kg^{-1}min^{-1}$)	Flow mediated dilation (%)
Pea	141 ± 7.5	79 ± 4.0	89 ± 5.4	1.2 ± 0.08	12 ± 0.8	1.6 ± 0.2
Nitrate	148 ± 5.8	86 ± 4.5	84 ± 4.7	1.2 ± 0.1	13 ± 1.1	1.5 ± 0.2
Control	148 ± 5.8	86 ± 4.5	83 ± 4.5	1.3 ± 0.1	13 ± 1.1	1.4 ± 0.1
Beet	152 ± 7.8	80 ± 3.2	77 ± 6.0	1.2 ± 0.1	13 ± 1.1	1.6 ± 0.1
Nitrite	152 ± 7.8	80 ± 3.2	80 ± 6.3	1.2 ± 0.1	13 ± 1.2	1.8 ± 0.2

Values shown as mean ± SEM, n=8. No significant difference, $P > 0.05$; 1-way ANOVA with LSD post-hoc test. Diets are shown in order of decreasing nitrate level.

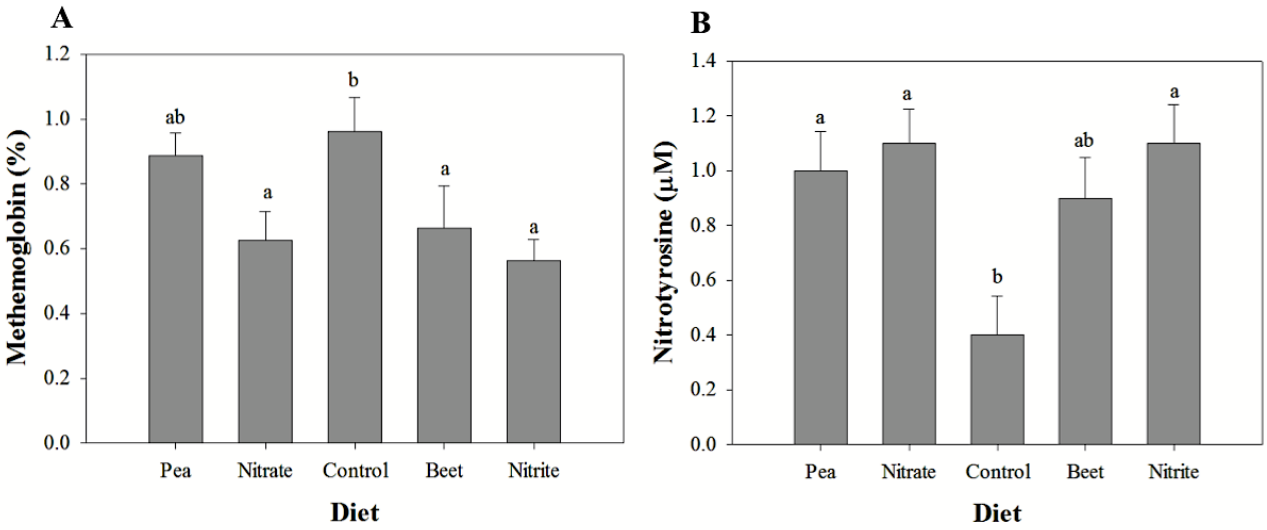
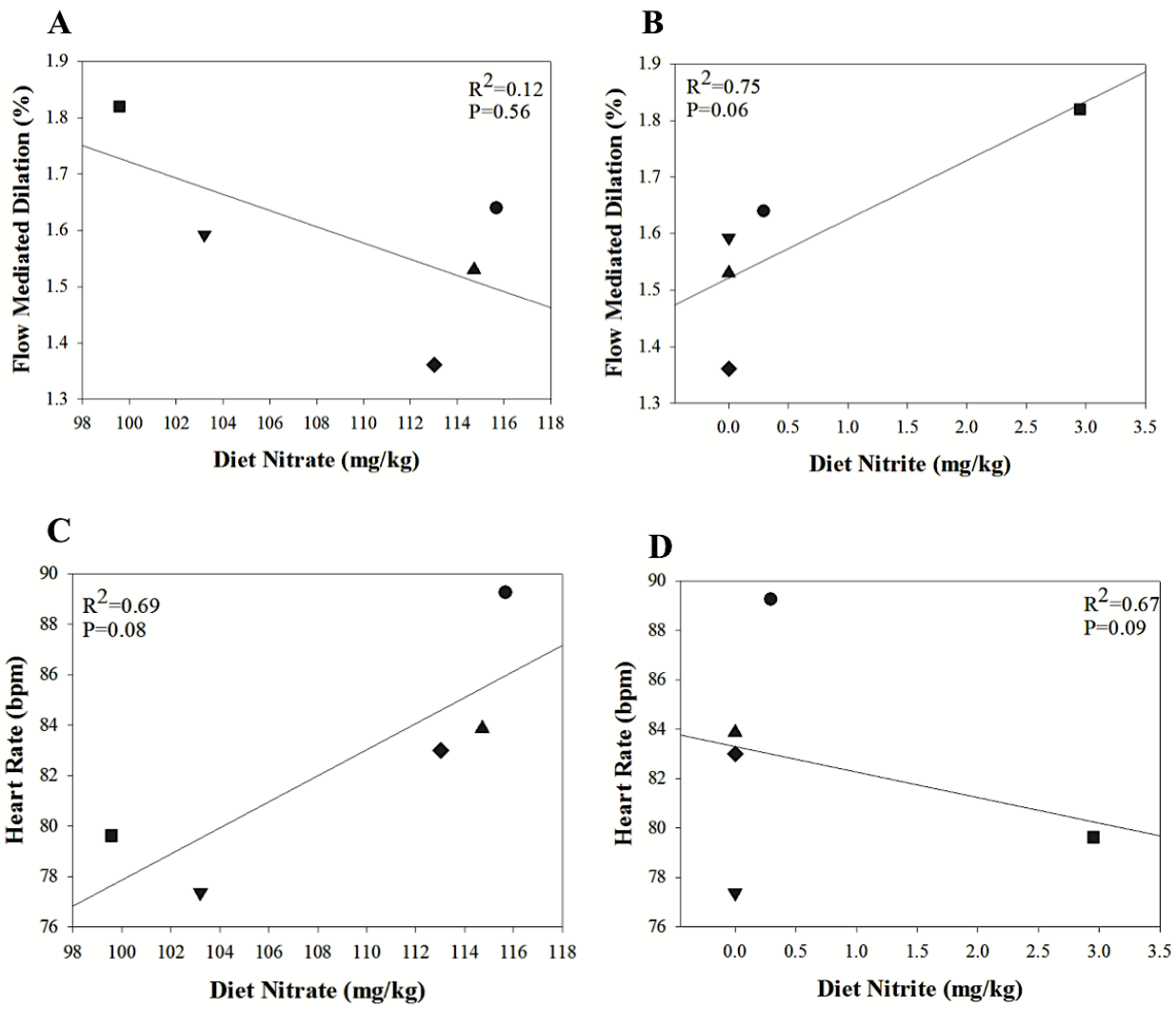


Figure 4.1 Biomarkers of toxicity in dogs fed test diets. Whole blood methemoglobin (A) and plasma nitrotyrosine (B) was measured in blood collected from dogs fasted overnight after six days of feeding each diet. Values shown as mean ± SEM, n=8. Values with superscripts without a common letter differ, $P < 0.05$; 1-way ANOVA with LSD post-hoc test. Diets are shown from left to right in order of decreasing nitrate level.

4.5.5 Regressions

Relationships between either dietary nitrate or dietary nitrite levels are shown relative to cardiovascular endpoints in dogs fed different test diets for six days in Figure 4.2. First, there was a weak positive relationship between dietary nitrite and flow mediated dilation (simple linear regression; $R^2>0.6$), while there was no significant relationship between diet nitrate and flow mediated dilation ($R^2<0.6$). Similarly, heart rate showed a weak positive relationship with dietary nitrate ($R^2>0.6$), but a weak negative relationship with dietary nitrite($R^2>0.6$). While there was no significant relationship between stroke volume and dietary nitrite ($R^2<0.6$), stroke volume showed a weak positive relationship with dietary nitrate ($R^2>0.6$). Lastly, there was a significant, positive relationship between diet nitrite and nitrotyrosine ($R^2>0.6$).


A
Flow Mediated Dilation (%)
Diet Nitrate (mg/kg)
R2=0.12
P=0.56
B
Flow Mediated Dilation (%)
Diet Nitrite (mg/kg)
R2=0.75
P=0.06
C
Heart Rate (bpm)
Diet Nitrate (mg/kg)
R2=0.69
P=0.08
D
Heart Rate (bpm)
Diet Nitrite (mg/kg)
R2=0.67
P=0.09

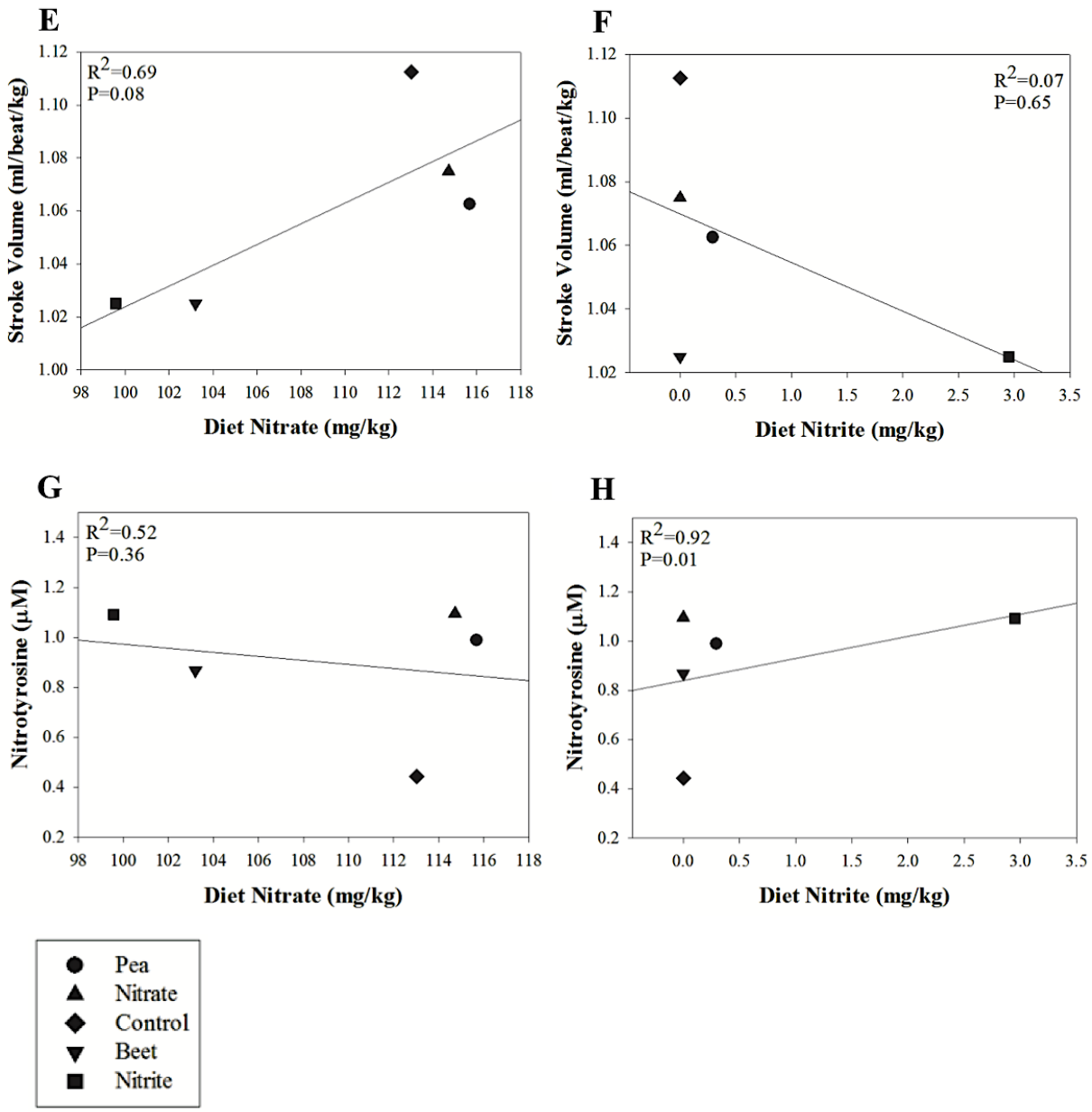


Figure 4.2 Simple linear regression showing dietary nitrate and nitrite relationships in dogs fed test diets for six days. (A) No relationship between diet nitrate and flow-mediated dilation. (B) Weak positive relationship between diet nitrite and FMD. (C) Weak positive relationship between heart rate and diet nitrate. (D) Weak negative relationship between diet nitrite and heart rate. (E) Weak positive relationship between diet nitrate and stroke volume. (F) No relationship between diet nitrite and stroke volume. (G) No relationship between dietary nitrate and plasma nitrotyrosine. (H) Positive relationship between dietary nitrite and plasma nitrotyrosine. Significant relationship determined as $R^2>0.6$.

4.6 Discussion

The present study examined the effects of nitrate and nitrite, both from plant sources or from pure chemicals, in canine diets on cardiovascular function, methemoglobin and oxidative stress. Nitrate and nitrite are nitrogenous compounds with potential for both therapeutic and toxic qualities. An inadvertent key finding of this study is proof that commercial feed ingredient suppliers, either intentionally or unintentionally, add nitrate and/or nitrite to their products, resulting in nitrogen doping. Canine diets in this study were formulated at 18% percent protein, at the minimum AAFCO protein requirement for dogs (AAFCO 2013). However, after proximate analysis, apparent crude protein content of all test diets was 31-37% higher than what was formulated. Crude protein is measured using the Kjeldahl method, whereby total nitrogen is quantified based on heating a feed sample in sulfuric acid, in order to oxidize the sample and extract the nitrogen (Michałowski et al. 2012). Since the Kjeldahl method is just as a summary of total nitrogen in a sample, nitrogenous compounds such as nitrate and nitrite would test positive for apparent crude protein (Bowman et al. 1988). Based on the findings of nitrate analyses of our major feed ingredients, high nitrate in the chicken meal that was used as the primary protein source in all of our test diets, may have contributed to crude protein in the diets being 27-37% higher than what was originally formulated. Nitrate has the ability to be used as a non-protein nitrogen doping agent, similar to the way melamine was used to boost apparent crude protein content in 2007 (Xin and Stone 2008). Melamine, a nephrotoxic compound used in plastics manufacturing, was incorporated into pet food in the form of tainted wheat germ. The toxic melamine was added to the wheat germ by Chinese distributers to boost the apparent crude protein content of ingredients that was then unknowingly used as an ingredient by a large number of pet food manufacturers (Xin and Stone 2008). This incident resulted in the recall of several pet food brands and the deaths of many pets across North America (Vail et al. 2007). In the current study, while nitrate may have contributed to the unexpectedly large apparent crude protein, the difference in formulated versus measured crude protein content is too large to be solely from the addition of nitrate. In order to confirm the true protein content, additional feed and ingredient samples need to be analyzed using total amino acid analysis since this is the most accurate method to measure true protein content (Chang and Zhang 2017)

Supplementary nitrate and nitrite was incorporated into the diets either from organic or inorganic sources. Organic plant nitrate sources were beet root or whole ground peas. Beet pulp

is already used extensively in the pet food industry as a source of slowly digestible fibre (de Godoy et al. 2013). In contrast, beet pulp and extract has been investigated as an antihypertensive natural dietary supplement in human health (Kröger et al. 2017). Beet pulp used in pet food contains 19-58 mg/kg nitrate, which is much lower than what is used medicinally in human health. When beet root is used medicinally in humans, it contains the whole beet and not just the dried, fibrous pulp. Raw beet juice is the part of the beet containing the highest concentration of nitrate, approximately 251-978 mg/kg (Antczak-Chrobot et al 2018). The combination of the fibrous pulp and beet juice contains enough concentration to improve vascular distensibility in humans (Kukadia et al. 2019). This means that the beet root used in pet food and the present study are likely not high enough in nitrate to produce a vascular effect. In contrast, a study by Bahadoran et al. (2016) examined the concentration of nitrate and nitrite in different vegetables, fruits, grains, and legumes. Of the legumes tested, cowpeas, chickpeas and green peas contained the highest concentrations of nitrate and nitrite, with a mean nitrate concentration of 37 mg/kg and a mean nitrite concentration of 0.73 mg/kg. In the present study, the concentrations of both nitrate and nitrite were lower than these values reported in the literature. This could be a result of the beets and peas being grown under low nitrate/nitrite conditions. There are several factors that can influence the uptake and storage of nitrate into plant products including soil and weather conditions (Scholefield et al. 1993). Plants often absorb nitrate more rapidly if the soil is treated with fertilizer (Malhotra 2016). The beet pulp and peas incorporated in the present study may have only been exposed to low concentrations of fertilizer during the growing period, reducing the absorption of nitrate and nitrite. Pulse crops, such as peas, are nitrogen fixing plants which take nitrogen from the atmosphere and convert it into nitrogenous compounds like ammonium, nitrate and nitrite in the soil (Townley-Smith et al. 1992). In Saskatchewan, pulse crops are grown on rotation with nitrogen requiring crops like canola and cereal crops. This means that when the pulse crops are planted, they are planted in soils containing low nitrogen compounds (Adderley et al. 2006). Therefore, there would be limited nitrate and nitrite for the pulses to absorb as they grow. Furthermore, yellow peas were the species of peas used in the present study. Lower nitrite and non-detectable nitrate values for the peas used in our study may indicate a species difference for the nitrate and nitrite content of peas. However, there has been no research to support the normal nitrate and nitrite content of yellow peas.

Nitrate and nitrite are volatile compounds, which are easily converted under specific conditions. The diets in the present study were formulated with an ingredient including 115mg/kg nitrate, as well as supplementary nitrite or nitrite ingredients. However, after analysis it was determined that nitrate and nitrite in the whole diet was slightly less than what was supplemented. Nitrate and nitrite were likely converted into other nitrogenous compounds during formation of the diet. A study by Honikel 2008, examined the use of nitrate and nitrite in curing meat products. It was determined that with heating, there was a disappearance of nitrate and nitrite from the cured meat. It was determined that there was a significant loss of nitrate and nitrite in pork when heated at 80°C and 70°C when compared to sample that were not heat treated. The loss of nitrate and nitrite in the diets in the present study could be a result of drying the feed at 65°C. Ultimately, if manufacturers wish to incorporate nitrate and nitrite into their diets, they will need to account for loss during processing. In contrast, this means that the high nitrate chicken meal used in the study may also have had even higher nitrate than the detected 115.4 mg/kg before cooking and rendering by the feed manufacturer. This further prompts the need for FDA regulation of nitrates in pet food. Alternatively, some of the excess nitrogen in the chicken meal in the current study may have come from other sources of non-protein nitrogen, but the identity of that source remains unknown.

Inclusion of 30% whole ground peas in canine test diets in the current study decreased both protein and fat digestibility, with the pea diet lower than all other diets. A 1977 review outlines the problems with legume protein digestibility in dogs. The review analyzed the protein digestibility of a variety of legumes, with raw and cooked peas having the lowest protein digestibility at 59%. Dogs fed split peas also had the greatest fecal nitrogen excretion, with fecal nitrogen being 21.2% of intake. The review concluded that the main reason for low *in vivo* protein digestibility is that raw legumes contain a large amount of antinutritional factors, specifically trypsin inhibitors and hemagglutinin compounds (Bressani and Elias 1977). These trypsin inhibitors may be destroyed by extrusion of the pet food or fermentation of the peas (El-Hady and Habiba 2003). The diets used in the present study were not extruded or fermented. The test diets were instead were cold-pelleted, but were dried in an oven, but only at 65°C. Our results suggest higher than 65°C is needed to degrade trypsin inhibitor or other anti-nutritional factors and likely accounts for the lower protein digestibility. However, why the fat digestibility was also lower in dogs fed a diet with raw pea meal is unclear since no literature could be found

to explain this. Total tract digestibility may have also been negatively influenced by the diarrhea observed in dogs fed the test diets. Protein and fat digestibility may be reduced in animals exhibiting symptoms of soft stool and diarrhea as a result of increased gastric emptying and motility. This reduces the time for absorption of essential nutrients in the small intestine (Wennogle et al. 2016). The high levels of nitrate or possibly other unknown non-protein nitrogen compounds in the test diets may have contributed to the presence of diarrhea in dogs fed the diets. With 75% of the dogs in the trial presenting with soft stool for the six days each that all diets were fed, the cycling of nitrate compounds may have resulted in the production of free radical species in the gastrointestinal intestinal system (Keshavarzian et al. 2003). Inflammation of the small bowel and diarrhea, associated with supplementary nitrate, may have been an unintentional gastrointestinal toxicity observed in this study.

One of the key findings of the study was that with the addition of inorganic nitrate and nitrite, we failed to see higher levels of methemoglobin than the control, as originally predicted. Dogs with methemoglobinemia do not begin to show signs of hypoxia until >20% methemoglobin (McKenna et al. 2014). Methemoglobin in all diets were at subclinical levels <2% in the current study. In fact, methemoglobin was observed to be lowest in dogs fed the nitrate and nitrite diets in the current study. Although it was not measured in this study, the lower methemoglobin may have been a result of a stimulation in the enzyme methemoglobin reductase. Methemoglobin reductase is the NADH-dependent enzyme responsible for reducing the oxidized ferric iron in methemoglobin back into the ferrous state (Ha and Bhagavan 2011). Unlike hemoglobin oxygenase, an enzyme which catalyzes heme breakdown into iron, biliverdin, and carbon monoxide (Rogers et al. 2003), methemoglobin reductase activity is not well studied. A 2018 study examined methemoglobinemia recovery in rainbow trout. There was a positive trend in methemoglobin and deoxygenated red blood cells reduction observed in trout exposed to nitrite in the aquatic environment. It was determined that the positive recovery was the result of methemoglobin reductase stimulation as a response to low oxygen saturation (Jensen and Nielsen 2018).

Nitrotyrosine is considered a biomarker of oxidative stress and an indicator of chronic inflammation (Kaur and Halliwel 1994). However, plasma nitrotyrosine can also be viewed as an indicator of nitric oxide production in the presence of oxidative stress (Van Der Vliet et al 1996). Nitric oxide is a reactive nitrogen species produced by the reduction of nitrogenous compounds

such as nitrate and nitrite. Nitric oxide reacts with superoxide anion to form peroxynitrite. As a reactive molecule, peroxynitrite covalently modifies many macromolecules to cause damage, but has a high affinity for modifying tyrosine residues on cellular proteins, which in turn results in the production of nitrotyrosine (Boelsterli 2002). The nitrotyrosine results of the present study support the hypothesis that canine diets containing supplementary nitrate and nitrite influence the production of nitric oxide. Specifically, as nitrite levels increased in the test diets, plasma levels of nitrotyrosine increased proportionally. This disagrees with studies examining long-term nitrate and nitrite supplementation in rats that found decreased oxidative stress (Norouzirad et al. 2019) A study by Gheibi et al. 2018 studied the effect of long-term nitrate supplementation on inflammation associated with type II diabetes in rats. Results showed that with chronic nitrate supplementation, there was an increase improved glucose tolerance, insulin resistance, and dyslipidemia, coupled with a decrease in oxidative stress and inflammation. The results of the nitrotyrosine analysis in our study show that serum nitrotyrosine could be used as an indicator of nitric oxide production as a result of short-term dietary nitrite supplementation. The analysis of C-reactive protein (CRP) in the plasma did not reveal an indication of inflammation in the dogs. Levels of CRP remained below a level detected by the ELISA assay. CRP is an acute phase inflammatory protein produced by the liver and is used as a major indicator of adverse cardiovascular risk in humans (Parrinello et al. 2015). However, in dogs the methods for measuring CRP are not as highly sensitive as the measurements used in humans, with canine CRP kits generally only being sensitive and reliable in animals with serious inflammatory conditions or infections where CRP would be much higher (Selting et al. 2015). This could explain the non-detectable levels of CRP in the present study.

We predicted that with supplemental nitrate and nitrite, it should correlate to high flow-mediated dilation, but we saw no differences in any cardiovascular end-points in dogs after being fed different test diets for six days. All cardiovascular measurements were within the normal range for dogs (Hopper 2009). Despite no difference in ANOVA analyses, we did detect some influences of nitrate and nitrite concentrations in the diets on cardiovascular function through regression analyses. The positive relationship between dietary nitrite and flow mediated dilation supports our hypothesis and coincides with nitrite findings in human studies, where dietary nitrite improved endothelial function and vascular distensibility through conversion into nitric oxide (Machha and Schechter 2011). A study by Stokes et al. 2009 used mice to observe the

reversal of endothelial dysfunction in animals exposed to oral nitrite in drinking water. It was noted that mice with hypercholesterolemia did not develop diet-induced arteriolar dysfunction and exhibited signs vasorelaxation to acetylcholine when dosed with 150mg/l water nitrite. In contrast, the present study indicated that dietary nitrate fed to dogs for one week has no influence on blood pressure or flow-mediated dilation. Similar to a study by Bahra et al. 2012, where it was observed that inorganic nitrate ingestion in humans did not alter flow mediated dilation. However, Bahra et al. also noted that after ingestion of 8 mmol of potassium nitrate for 28 days, human subjects did show significant reductions in systolic blood pressure. With a feeding period longer that six days, lower blood pressure may also have been observed in the dogs used in the present study.

In the current study, dietary nitrate and nitrite had opposite effects on heart rate. Similar to the positive association between dietary nitrate and heart rate found in this study, Tamargo and Delpon state in their book on *The Pharmacology of Nitrates,* that a high nitrate dose has the potential to create tachycardic conditions. This was attributed to a baroreceptor reflex, associated with hypotension as a result of vasodilation (Tamargo and Delpon 1997). Our study showed a similar relationship between nitrate and stroke volume, where stroke volume increased with increasing dietary nitrate. Nitrates have the ability to improve coronary blood flow to the heart, causing a subsequent increase in stroke volume (Abrams 1996). An increase in stroke volume and heart rate would eventually result in an increase in cardiac output. With more blood being pumped out of the heart per minute, it may result in elevations in arterial pressure (Guyton 1981). Conversely, this study showed that dietary nitrite has a negative influence on heart rate. Nitric oxide has been shown to inhibit beta adrenergic activity in dogs (Hare et al. 1995). Hare et al. studied parasympathetic modulation beta-adrenergic myocardial contractility in response to nitric oxide in dogs. Their results suggested that nitric oxide may mediate vagal inhibition as observed by a negative ionotropic response, with beta-adrenergic stimulation by dobutamine. A 2011 study demonstrated similar results in rats given an acute dose of 1, 3 and 5 mg/kg of intravenous sodium nitrite, showing a dose dependent decrease in heart rate. The bradycardia observed over 1±0.5 hr was attributed to an energy saving mechanism, as a result of hypoxic conditions (Schumilova et al. 2011). Another study by Rastaldo et al. 2007 reviewed the dose-dependent response of nitric oxide on myocardial contractility. The authors stated that with low doses of nitric oxide there is an increase in myocardial contractility, as a result of low production

of cGMP in myocardial cells and promotion of calcium release to stimulate contractility. Conversely, it was stated that high doses of nitric oxide induce greater cGMP production, causing cardiac depression as a result of calcium blockage. In relation to the present study, dietary nitrate may not have been as readily converted to nitric oxide as dietary nitrite did, allowing for the positive relationship observed with nitrate and the negative relationship observed with nitrite.

4.7 Conclusions

The overall findings of this study demonstrate that nitrate and nitrite can act as a doping agent and boost the apparent crude protein content of pet food, without affecting digestibility in most cases. There were cardiovascular differences among dogs after feeding dietary nitrate and nitrite from organic and inorganic sources, for 6 days, while biomarkers of toxicity remained at subclinical levels. The results of this study show that dietary nitrite is more likely to exert a therapeutic effect to reduce blood pressure and improve cardiac conditions with chronic feeding in dogs. In contrast, dietary nitrate may also have some ability to improve vascular endothelial function, but with less efficiency compared to dietary nitrite. Furthermore, with prolonged with high dietary nitrate exposure, the effects of improved vascular function may be counteracted by increases in stroke volume and heart rate. Ultimately, this study illustrates that dietary nitrite may be more beneficial in improving cardiovascular function in dogs than dietary nitrate.

5.0 OVERALL DISCUSSION

5.1 Summary of conclusions

The research completed in this thesis examined protein quality in pet food. The main objective of this study was to investigate the inclusion of toxic nitrogenous compounds in pet food and how non-protein nitrogen affects nitrogen utilization and cardiovascular function in dogs and cats. The results of the first study revealed that there are protein differences in commercial pet foods and different pet food brands contain different concentrations of toxic non-protein nitrogen sources. Therefore, the overall hypothesis that protein quality in commercial pet foods will be similar was rejected, as there were differences observed in digestibility and non-protein nitrogen content. Results of the second study showed that dietary inclusion of nitrate and nitrite in pet food have different effects on cardiovascular function. The second overall hypothesis that canine diets containing increasing concentrations of nitrate and nitrite would lower blood pressure was rejected, as only nitrite positively influenced flow mediated dilation and no change in blood pressure was observed.

The following is a short summary of the most significant findings from each of the studies in this thesis:

1. Study 1 determined that price of commercial pet food reflected crude protein content, with diets higher in price containing greater crude protein.
2. Study 1 found that commercial diets highest in price also contained the highest concentrations of toxic non-protein nitrogen compounds.
3. Study 1 showed that commercial diets do not produce the same levels of toxic biomarkers.
4. Study 2 demonstrated that nitrate can be used as a doping agent to improve apparent crude protein content.
5. Study 2 showed that diets containing an inclusion of ≤115 mg/kg inorganic nitrate and ≤20 mg/kg nitrite can lower methemoglobin levels.
6. Study 2 found that there were differences in cardiovascular function between increasing dietary nitrite and nitrite, nitrite showing improvements in vascular distensibility and nitrate being more cardiotoxic.

The results of Chapter 3 indicate that pet owners do not need to spend money on the highest priced pet food, when considering protein. The moderate and low priced diets did not have any negative effects on cardiovascular function, protein utilization or levels of toxic biomarkers. Moreover, moderately priced diets contained lower concentrations of non-protein nitrogen compounds.

Results of Chapter 4 do not support the theories of previous studies which state that dietary nitrate can be used therapeutically to improve vascular endothelial function (Webb et al. 2008). This study showed that in dogs, additional dietary nitrate did not lower blood pressure or improve vascular distensibility. Instead, increasing dietary nitrate was positively associated with increased heart rate and stroke volume. These results indicate that dietary nitrate should not be used as a vasodilatory agent in animals suffering from hypertension. Alternatively, dietary nitrite showed some therapeutic potential, with increased flow mediated dilation and lower heart rate.

5.2 Strengths and limitations

A strength of study one was the use and comparison of two companion animal species; dogs and cats. The methods used were not as optimal and accurate for use in cats as they were in dogs. However, use of both species allowed for better investigation of commercial pet food brands, with a reduced species bias between products. Using both dogs and cats also allowed for better examination of handling and absorption of nitrate and nitrite between the two species. Commercial diets have similar formulation for both cats and dogs. While dogs and cats share some similarities, there are some major differences in their digestive physiologies and metabolism (Willard et al. 2002). Cats have greater difficulty digesting plant protein than dogs, yet feline diets still possess significant plant protein content (Case et al. 2010). Thus, it was in the best interest of the study to include both species to analyze the protein utilization in both species, even though commercial dog and cat food are so similar.

Another benefit to the first study was the use of AAFCO approved brands. This allowed for standardization for selection of different diets. With diets containing similar macronutrient profiles, it allowed for a better comparison of protein in pet foods with regard to price. The similarity between selected diets also reduces the likelihood that differences observed between endpoints was a result of protein content and not an external confounding factor. Even though the commercial diets selected had similar protein sources (chicken being the major protein

source), there still may be differences in rearing, rendering and processing of the protein source (Hendriks et al. 1999). These factors may influence the handling of the protein product in the companion animal to which it is fed (Tran et al. 2008).

A strength of the second study was the comparison of both plant and inorganic sources of nitrate and nitrite in canine diets. This allowed for the opportunity to examine the bioavailability of these nitrogenous compounds in plant sources. Since canine digestive physiology is similar to that of humans, the dogs in this study could also be used as a model for studying the absorption and utilization of dietary nitrate and nitrite. Dietary nitrate as a vasodilatory agent in humans is most commonly utilized in the form of beet juice (Siervo et al. 2013). This study allowed for investigation of whether beet juice is the most efficient delivery system for nitrate or if inorganic nitrate should be used instead.

An additional benefit to the second study was the behaviour of the dogs used in the study. The moderate temperament of the beagles allowed for minimal variability in cardiovascular endpoints as a result of environmental factors. Cardiac endpoints in animals, particularly heart rate can be easily influenced by simple stressors such as handling, noises or lighting (Gácsi et al. 2013). The beagles used in the study were calm during handling, without the use of sedation and did not show resistance to procedures (in fact they enjoyed test days because of the extra attention and cuddles). This allowed for accurate cardiac and plasma measurements. Similar studies using non-sedated animals have shown difficulties in gathering accurate heart rate data (Von Borell et al. 2007). The use of calm animals, without sedation resulted in more accurate, real-world values.

The primary limitation of both studies was the small sample size. The use of only 4-8 samples resulted in significant variability. With such a small sample size, it was difficult to eliminate outliers and Type II statistical error is likely for many of the end-points we assessed. The use of additional animals would have reduced variability and potentially revealed more significant results. Similar human studies examining effects dietary nitrate on the cardiovascular system often use >50 test subjects (Velmuruganet et al. 2015). This may be why these studies often observe more significant changes in cardiovascular endpoints after manipulation of dietary nitrate or nitrite intake.

Another limitation to the studies was the short feeding period (six days) of the diets. A long-term feeding trial may have revealed more significant results than the seven days that were

used. Certain non-protein nitrogen compounds, like nitrate and nitrite, produce reactive oxygen species. Since the diets in both Chapter 3 and 4 did not contain nitrate and nitrite concentrations that were acutely toxic, a longer feeding period may have resulted in greater indications of oxidative damage and inflammation (Kina-Tanada et al. 2017). This would have been observed as greater overall differences and significance in biomarker assays and cardiovascular assessment. However, budget and limited time for access to the research beagles due to other projects prevented examination of longer feeding.

5.3 Future work

There is ongoing debate among pet owners over which pet food provides pets with the best nutritional value. The results of Chapter 3 indicate that there are differences in protein quality and utilization between commercial pet foods. A beneficial next step would be to conduct a total amino acid analysis. It has been speculated that taurine may actually be a semi-essential amino acid in dogs (Serrill et al. 2016). In 2019, the FDA sent out a statement regarding a potential link between dilated cardiomyopathy and grain free dog food. According to FDA, there has been case studies of dogs diagnosed with dilated cardiomyopathy being taurine deficient and being fed pulse-based diets (FDA 2019). However, there is a certain amount of controversy surrounding this statement as it is only based on case studies and incidence of dilated cardiomyopathy is also more prevalent in certain large breed dogs. Future work for this study could include a taurine analysis of the commercial diets and paired with a regression analysis to screen for early indicators of dilated cardiomyopathy.

In order to definitively determine a superior pet food brand, similar studies must be performed for all macronutrients. Fat and fiber quality and utilization should also be examined in the same pet food brands. Since protein only makes up 18-38% of the selected diets, fat and fibre also greatly influence the overall physiological function and well-being of the animal.

As stated above, the second study would benefit from long term feeding trials. A 2018 study similar to the present study fed dietary nitrate to rats for two months. A compensatory mechanism was observed with a reduction in markers of oxidative stress (Gheibi et al. 2018). However, nitrate and nitrite are potential carcinogens and if fed long term could cause cancer formation (Grasso 1983).

5.4 Final conclusions

The results of this study provide a greater insight on the options available in the pet food industry. All of the commercial diets tested provided cats and dogs with adequate nutritional value and did not cause any immediate toxic effects. It was determined that consumers do not need to consider protein, when debating prices of commercial pet food. As a primary nutrient in required by cats and dogs, it is important for consumers to consider the source of protein included in pet food. Different types of animal protein, as well as the incorporation of excess plant protein have the ability to alter nitrogen utilization in pets. Furthermore, while non-protein nitrogen sources were detected in the commercial diets tested, nitrate and nitrite concentrations were also not high enough to have any cardiovascular therapeutic potential or to cause immediate toxic effects.

Dietary nitrate is used as a vasodilatory agent in human medicine. The results of this study indicate that dietary nitrate as a plant and/or inorganic source should not be used in veterinary medicine. Increases in dietary nitrate showed early indicators, which could potentiate hypertensive conditions. Meanwhile, low doses of inorganic nitrite have therapeutic potential as an antihypertensive agent to be used in dogs.

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